**Universidade de Aveiro** Departamento de Biologia
**Ano** 2016

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# Contaminação bottom-up em sistemas marinhos – Níveis tróficos modelo para avaliar o fluxo de cádmio em organismos marinhos

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Investigadora Auxiliar do Departamento de Biologia e CESAM-Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro, e co-orientação do Doutor Ricardo Jorge Guerra Calado, Investigador Principal do Departamento de Biologia e CESAM-Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro e Professor Doutor Amadeu M.V.M. Soares, Professor Catedrático do Departamento de Biologia e CESAM-Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através de uma bolsa de doutoramento atribuída a Maria Pavlaki (SFRH/BD/70906/2010).

To the loved ones who left without me getting to say goodbye

**o júri**

presidente

Prof. Doutor Armando da Costa Duarte
Professor Catedrático do Departamento de Química da Universidade de Aveiro

Prof. Doutor Henrique Manuel Roque Nogueira Cabral
Professor Catedrático do Departamento de Biologia Animal e MARE da Faculdade de Ciências da Universidade de Lisboa

Prof. Doutor Cornelis Adrianus Maria van Gestel
Professor Associado do Departamento de Animal Ecology, Faculty of Earth and Life Sciences, Vrije Universiteit of Amsterdam

Doutora Rosa de Fátima Lopes de Freitas
Investigadora Auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro

Doutora Sara Calçada Novais
Investigadora em Pos-Doutoramento do MARE e do Instituto Politécnico de Leiria

Doutora Susana Patrícia Mendes Loureiro (orientadora)
Investigadora Auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro

**agradecimentos**

During the last 4 years of this work, so many have participated, in one way or another, to make it happen that the least I could do is express my eternal gratitude.

First and foremost, my supervisors without whose support none of these would have been possible. Susana, words cannot express how grateful I am to you, not only as my supervisor but as a friend and “promising psychologist” too. Your constant help and motivation, professionally and personally, during not only the 4 years of the PhD but the last 8 years of working with you, keep me moving forward. Thank you for your help, friendship, patience and the occasional (and well deserved) scolding to keep me up to my toes. Ricardo, thank you for accepting me in your lab, for your patience and constant availability in solving my doubts every time I was lost in the “ecology stuff”. Honestly, I don’t think I ever found my way out. Professor Amadeu, almost 10 years ago and after countless emails, you helped me take the decision to study and work at UA. Thank you for had it not been you, I wouldn’t be here right now.

A special thank you to all of the people who, one way or another, took part in the completion of this work and a huge thank you to Dr. Kees van Gestel for his constant and tireless help with the toxicokinetic models and for, along with Rudo, making me feel welcome during my stay in Amsterdam.

To Abel, for the continuous help and patience in the lab and those endless conversations about work and everything.

To all the friends and colleagues, here in Portugal that keep making me feel like home ever since I got here. For that pat in the shoulder, for that hug, for all the good words that keep me going every time I miss home or feel sad. Grateful for having so many good people in my life!

A gigantic thank you to Rui and Fabi (you go along with family), for the coffees, wines, finos, petiscos, laughs, cries, late nights, stressful and less stressful moments I keep having with you! Looking forward to more of these! Twin, you know that without you I wouldn’t’ have made it till the end!

To my family who even far away, always close to my heart. For their unconditional love and understanding, constant support and inspiration even through the worst times and for making me who I am today. Thank you!

Finally, to Ricardo! For your love, understanding, patience, companionship and support you keep showing to me these last years. Thank you for joining in on my weirdness!

**palavras-chave**

Cádmio, *Acartia tonsa*, *Palaemon varians*, *Hediste diversicolor*, *Solea senegalensis*, bioacumulação, níveis tróficos.

**resumo**

O cádmio é considerado um dos metais mais tóxicos para organismos aquáticos, podendo ocorrer naturalmente no ambiente em concentrações muito baixas, devido a processos naturais (e.g., erupções vulcânicas, erosão da crosta natural) mas também devido a atividades antropogénicas, como a atividade mineira. Como um subproduto da exploração mineira de zinco, o cádmio pode ser libertado para o ambiente aquático através de lixiviação ou escorrências. O cádmio é um metal não essencial para os organismos mas, mesmo em concentrações relativamente baixas, pode ser tóxico, provocando efeitos adversos devido à sua elevada tendência para bioacumular. Neste contexto, o objetivo deste trabalho foi estudar a transferência de cádmio em diferentes modelos de níveis tróficos marinhos. O estudo foi dividido em quatro etapas: i) avaliar a eco- e genotoxicidade de cádmio em três espécies marinhas, representando diferentes níveis tróficos marinhos ii) determinar a bioconcentração de cádmio por *Acartia tonsa* sob diferentes condições ambientais, tais como pH, salinidade e temperatura, iii) a avaliação de toxicocinética de cádmio pelo camarão estuarino *Palaemon varians* sob três vias de exposição diferentes: água, alimentação e água + alimentação, e iv) avaliar os padrões de bioacumulação de cádmio no linguado *Solea senegalensis* como consumidor final, e os possíveis riscos e implicações do consumo da fração edível de camarões e peixe que pode ter para a saúde humana, após a exposição a cádmio. Foi observado que a toxicidade de cádmio é influenciada pela sua especiação. A maior sensibilidade ao cádmio foi observada em *A. tonsa* tendo como parâmetro mas sensível o Índice de Desenvolvimento Larvar (LDR). O cádmio induziu danos no ADN de todas as espécies utilizadas. A bioconcentração de cádmio por *A. tonsa* é fortemente afetada por diferentes condições ambientais devido a processos biológicos. *P. varians* foi exposto a cádmio através de diferentes vias de exposição: água ou alimento ou água e alimento. A exposição simultânea de *P. varians* a água e alimento contaminado com cádmio mostrou que a concentração interna de cádmio foi maior quando comparada com as outras duas vias de exposição. Finalmente, mesmo uma exposição através de água e alimento contaminado (fornecido como *Hediste diversicolor*) não foi suficiente para que o peixe *S. senegalensis* atingisse um *plateau* na concentração interna de cádmio, sendo os 14 dias de depuração insuficientes para que os organismos depurassem totalmente a concentração interna que havia sido acumulada. Adicionalmente, foi encontrada uma maior concentração de cádmio no intestino de *S. senegalensis* quando comparada com os outros órgãos, e a constante de eliminação de cádmio no fígado foi inexistente. Os valores do Coeficiente de Perigo Alvo (THQ) e o Consumo Semanal Estimado (EWI) para o cádmio estavam abaixo dos níveis aceitáveis estabelecidos em regulamentos europeus para a fração edível de *S. senegalensis*, enquanto que para *P. varians* tanto o THQ quanto o EWI excederam os níveis aceitáveis estabelecidos.

**keywords**

Cadmium, *Acartia tonsa*, *Palaemon varians*, *Hediste diversicolor*, *Solea senegalensis*, bioaccumulation, trophic levels.

**abstract**

Cadmium is considered one of the most toxic metals to aquatic organisms. This naturally occurring metal is found in the environment in low concentrations due to natural processes, such as volcanic eruptions, natural crust erosion and also anthropogenic activities, such as mining and smelting. As a by-product of zinc mining, cadmium can reach aquatic environment through leaching or to rainwater runoff from the mine areas. It is a non-essential metal for organisms that even at relatively low concentrations can be toxic and may cause adverse effects due to its high bioaccumulation tendency. Considering this, the objective of this work was to study the toxicity and bioaccumulation potential of cadmium within different model marine trophic levels. To achieve this goal, this work was divided into four studies: i) assess the eco- and genotoxicity of cadmium to three marine test-species, representing different marine trophic levels, ii) determine the bioconcentration potential of cadmium in the calanoid copepod *Acartia tonsa* under different environmental conditions, such as pH, salinity and temperature, iii) evaluate the uptake and depuration kinetics of cadmium by the estuarine ditch shrimp *Palaemon varians* considering three different uptake routes: water, diet, water + diet and iv) assess the bioaccumulation patterns of cadmium in the Senegalese sole *Solea senegalensis*, a final consumer, and the possible risk and implications the consumption of the edible fraction of both shrimps and fish may bear to human health upon Cd exposure. We observed that the toxicity of cadmium is highly influenced by its speciation. Highest sensitivity to cadmium was observed by *A. tonsa* while the most sensitive endpoint was the Larval Development Ratio (LDR). Cadmium induced DNA damage to all species with increasing concentrations. The bioconcentration of cadmium by *A. tonsa* is strongly affected by different environmental conditions due to biological processes. The simultaneous exposure of *P. varians* to cadmium-contaminated water + diet showed that cadmium internal concentration was higher when compared to the individual pathways. Finally, by exposing *S. senegalensis* for 14 days through contaminated water and diet (supplied as *Hediste diversicolor*), with another 14 days of depuration phase, it was concluded that the 14 days of exposure were not enough for the fish to reach a steady state on cadmium internal concentration, and neither the 14 days of elimination were sufficient to cause total depuration of the accumulated cadmium in any of the organs. Moreover, a higher concentration of cadmium was found in the intestine of the fish when compared with the rest of the organs, and the elimination rate constant of cadmium in the liver was nule. The Target Hazard Quotient (THQ) and the Estimated Weekly Intake (EWI) values for cadmium for the edible fraction of *S. senegalensis* were below the acceptable levels set by the European Regulation while for the shrimps both THQ and EWI exceeded the acceptable levels established.

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# Chapter 1
# General Introduction

## 1.1. Metals in marine and brackish ecosystems

Marine pollution has been defined by the Joint Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP) (1991) as "*the introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) resulting in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of sea water and reduction of amenities*". Marine and brackish ecosystems are simultaneously affected by natural changing conditions (e.g. weathering and erosion or increase of phytoplankton) and human impact that introduce contaminants (e.g. metals, fertilizers, pesticides or polycyclic aromatic hydrocarbons) to aquatic systems through point or diffuse discharges from different sources (industrial, agricultural, ballast and bilge water discharges due to ship transportation or domestic waste discharges) (Costa et al., 2008; Fonseca et al., 2011; Goswami et al., 2014; Kirkelund et al., 2010). These ecosystems are characterized by complex environmental processes and are considered of great economic value due to their natural productivity (e.g. ecosystem biodiversity, nursery grounds to many species), as well as for their use to a number of anthropogenic activities (e.g. commercial harbours and aquaculture) (Fonseca et al., 2011; 2015). Such processes may be disrupted by chemical contamination that may prompt in poor water quality with deleterious consequences to the environment and subsequently to humans.

Throughout the years, a great number of research studies have registered the high temporal and spatial variability of metal concentrations in marine/estuarine ecosystems through the monitoring of water quality characteristics and/or the use of biological indicators. The highest concentrations of metals, such as mercury (Hg), cadmium (Cd) and lead (Pb) as potential pollutants, are commonly recorded close to coastal and estuarine environments of highly populated and/or industrialized areas (Fowler, 1990). Even though, metal concentrations in the open sea are considerably low, usually in the order of ng $L^{-1}$, and may vary between regions: e.g. lead concentration recorded in surface waters of the Pacific Ocean can reach 14 ng $L^{-1}$, while in the Mediterranean Sea they can be as high as 30-150 ng $L^{-1}$ (Fowler, 1990); cadmium concentrations can reach values up to 250 ng $L^{-1}$ in French and Norwegian coastal areas (World Health Organization, 2003), while heavily contaminated coastal and estuarine environments may present concentrations significantly higher, in the order of μg/L or even mg/L (e.g. up to 8.9 μg of Cd $L^{-1}$ or 17.8 μg of Pb $L^{-1}$ (Vicente-Martorell et al., 2009)). Marine organisms can uptake metals either from the water or the sediment through direct or indirect contact

and at certain bioaccumulation levels in the organism may bear adverse effects due to their high affinity to biological membranes (Directorate-General for Health and Food Safety and European Commission, 2013). Studies have shown that toxicity occurs when metal concentrations exceed the physiological levels at the organism's target site (Mai et al., 2012; Morcillo et al., 2016). Some of these metals are considered essential for the organism to maintain homeostasis (e.g. copper, zinc), while others are considered non-essential and when encountered in the environment can cause toxicity and endanger marine ecosystems (Barka et al., 2001; Nugegoda and Rainbow, 1989; Rainbow and White, 1989; Wood et al., 2012). As an example, at concentrations higher than the physiological ones, copper caused embryo- and genotoxicity to larvae of the Pacific oyster, *Crassostrea gigas*, due to DNA damage and strand excision, possibly as a result of free radicals by oxidative stress (Mai et al., 2012). Methylmercury (MeHg) in marine fish cells causes cytotoxicity by impairing the lysosomes while cell death by apoptosis was observed after exposure to $Cd^{2+}$, $Cr^{6+}$, $Hg^{2+}$, $Cu^{2+}$, $As^{5+}$ and $Pb^{2+}$ (Morcillo et al., 2016).

Metal concentrations in the marine environment are mainly dependent on speciation due to the complex formation with other metals and ions, which in turn end up being deposited in the sediment of the contaminated area. Sediments are considered the final sink of metals after entering the aquatic environment, as metals tend to precipitate and remain associated to the sediment (e.g. adsorbed to particulates) for a long period of time (Jakimska et al., 2011b). Highly metal-impacted estuaries that present increased metal concentrations in their sediment may represent a risk to the ecology and overall life support of the ecosystem, depending on the system dynamics, which will rule sediment particles (re)suspension. Examples of contaminated estuaries, such as Ria de Huelva (Spain) or the Seine estuary (France) are a few among others to mention. Cadmium, lead and arsenic element load in sediments of the Ria de Huelva (Spain) reaches up to 33.2, 1167 and 615 mg $Kg^{-1}$, respectively, showing total metal accumulation of cadmium and lead in the edible fraction of a benthic and a pelagic species to be within the maximum levels (EC, 2005) however, arsenic concentration in the muscle tissues of both fish was higher compared to levels set by international guidelines (Vicente-Martorell et al., 2009). Another example is the Seine estuary (France), a macrotidal estuary with a single channel that due to anthropogenic and industrial activities (approx. up to 30% of the French population inhabits the area around the estuary and river), receives daily high metal input from the Seine river, e.g cadmium and lead with a sediment load ranging from 0.9 to 7 mg $Kg^{-1}$ and 80 to 175 g $Kg^{-1}$, respectively while values measured in benthic fish tissues were up to 0.04 mg $Kg^{-1}$ for cadmium and 0.83 mg $Kg^{-1}$ for lead (Chiffoleau et al., 1994; Henry

et al., 2012; Lacoue-Labarthe et al., 2008)', well above the maximum established levels of 0.3 mg $Kg^{-1}$ for lead but within maximum acceptable limits for cadmium (EC, 2005). An attempt to connect external levels of exposure, in sediment/water column with internal levels of metal accumulation in tissues and possible early adverse effects should always be considered by ecotoxicologists (van der Oost et al., 2003).

## 1.2. Cadmium in the marine environment – speciation and bioavailability

Cadmium is considered one of the most toxic metals to aquatic organisms (Howard and Hacker, 1990) and is categorized under the Framework Directive 2013/39/EU from REACH as a priority hazardous substance, making this one of the main reasons supporting the selection of this metal as the model contaminant for the present study. It is considered a non-essential metal for the organism that, even at relatively low concentrations, can be toxic and promote adverse effects due to its high bioaccumulation tendency (Chandurvelan et al., 2013a; 2013b; 2012). It is a naturally occurring metal, which may originate from volcanic eruptions and natural erosion of the earth's crust, but the main sources of cadmium in marine and brackish ecosystems are related to anthropogenic activities, such as industrial (e.g. smelting and mining, electronic waste incineration) and agricultural processes (e.g. phosphorous fertilizers) (World Health Organization, 2003). Cadmium is usually found in association with zinc, as it is a by-product from ore mining, and can leach into aquatic environments through water runoffs (Agency for Toxic Substances and Disease Registry (ATSDR), 2012; Environment Programme, 2008). Reported concentrations of cadmium in the marine environment were 250 ng $L^{-1}$ in coastal areas, whereas higher concentrations ranging from 8.9 µg $L^{-1}$ (Vicente-Martorell et al., 2009) up to 16 mg $L^{-1}$ (Cao et al., 2009) were recorded in estuaries and densely inhabited areas, and have been associated with river input (Elinder, 1985; World Health Organization, 2003). Frazier (1979) reported concentrations of cadmium in marine organisms reaching values up to 54 and 140 µg $g^{-1}$ in fish and molluscs, respectively. The release of cadmium into the environment has been regulated by national and international authorities through existing actions and legislation, certainly not being limited to REACH, in order to control the potential contamination of aquatic ecosystems (OSPAR Commission, 2004).

Several studies are available on the effects of cadmium on marine organisms from different trophic levels and are used by regulatory parties to support the creation of laws and regulations concerning metal pollution. Marine and brackish environments have

always been a challenge to researchers addressing this topic due to the complex processes that metals undergo once in these ecosystem, as total or dissolved concentrations are not always indicative of the potential adverse effects they may produce (Campbel, 1995; Janssen et al., 2000). Therefore, it is paramount to consider metal speciation and bioavailability in ecotoxicology and risk assessment studies (Janssen et al., 2000; Paquin et al., 2002). Metal speciation in marine ecosystems depends greatly on water chemistry, being strongly influenced by salinity, temperature, pH, dissolved organic compounds, calcium and zinc concentration, redox potential, ionic strength and inorganic ligands (Amirthalingam et al., 2013; Engel and Fowler, 1979; Environment Programme, 2008; Frazier, 1979; Panda and Panda, 2002; Ray, 1984). Bioavailability is the degree to which a contaminant is available in the surrounding medium for the organism to uptake and subsequently cause a possible effect (Newman, 2014; Plette et al., 1999).

Speciation of cadmium is mainly influenced by the existence of chloride ions in seawater and bioavailability, as free ion concentration, and therefore toxicity, is inversely correlated to salinity due to complexation with chloride ions (Sunda et al., 1978). Zirino and Yamamoto (1972) demonstrated that cadmium remains strongly complexed with chloride ions over a wide pH range and that species distribution is not significantly changed. Cadmium cations will compete with other cations (e.g. $Ca^{2+}$, $Fe^{2+}$) for dissolved ligands, such as anions (e.g. $Cl^-$) or ligands in biological membranes (Di Toro et al., 2001; Newman, 2014; Paquin et al., 2002). Cadmium species will form either organic or inorganic complexes, with the first ones being organic matter (e.g. carboxyl and phenolate groups) and the second ones being inorganic species (e.g. $Cl^-$, thiol groups) (Newman, 2014). Once it has entered the marine environment, cadmium will mostly precipitate (mainly in the form of sulfides) and deposit in the sediment (Jakimska et al., 2011b).

Several studies have demonstrated that the uptake of cadmium by the organism is mainly influenced by the availability of the free metal ions rather than the concentration of the total dissolved cadmium and, as previously mentioned, it is highly dependent on physicochemical conditions (Burke et al., 2003; Engel and Fowler, 1979; Roast et al., 2001; Sunda et al., 1978). Mubiana and Blust (2007) demonstrated that uptake and elimination of cadmium by the marine bivalve *Mytilus edulis* increased with increasing temperature. Ali and Taylor (2010) presented data showing that cadmium internal concentration in *M. edulis* decreased with increasing salinity. Bjerregaard and Depledge (1994) reported that a reduced calcium concentration is the main mechanism behind the increase in cadmium internal concentration with decreasing salinity, as $Cd^{2+}$ competes with $Ca^{2+}$ for uptake sites. In accordance with the Free Ion Activity Model (FIAM), uptake

and toxicity of metals depends on the concentration of the free metal ion activity and its interactions with the organism (Morel, 1983). Later on and based on the FIAM and the GISM (Gill Site Interaction Model) model, the Biotic Ligand Model (BLM) was developed under the conceptual framework that mortality of an organism occurs when the concentration of the metal-biotic ligand complex reaches a critical level (Di Toro et al., 2001). The BLM is based on the hypothesis that the total metal concentration does not relate directly to the toxicity but rather the free metal ions competing with cations at the active site and possible transport sites into the body. Free ion concentration and free ion activity can be estimated based on the water composition in terms of elements, by using chemical equilibrium speciation models. Visual MINTEQ (Gustafsson, 2013) is one of those models/tools, available as a freeware programme, that incorporates biotic ligand interactions to allow the quantification and speciation of cadmium in order to better understand and evaluate the toxic effects and accumulation levels in the organism as well as to facilitate comparison between studies.

## 1.3. Ecotoxicity and genotoxicity of cadmium

As previously referred, there is a great need to better understand the bioavailability and the way metals interact with aquatic organism and marine and brackish environments. The concept of ecotoxicology incorporates the ecological and toxicological methods employed to analyse and assess the effects that pollutants may have in the environment, focusing on individual and subsequently at population level (Luoma, 1996). Those effects are usually reported based on the concentration measured in the surrounding media in relation to a percentage of the effect observed in a population (Ware, 1999). However, marine and brackish ecotoxicology, when compared to freshwater or soil ecotoxicology, is still at its infancy due to the complexity displayed by these ecosystems. Following or adapting existing International Guidelines for testing cadmium, several studies have recorded the ecotoxicity of cadmium to marine/estuarine organisms. Cadmium effects as an environmental contaminant have been assessed in aquatic ecotoxicology for the past 50 years and classic toxicological endpoints, such as mortality, reproduction, feeding behaviour or growth have been the main reference points for risk assessment (Newman, 2014). Once in the marine environment, cadmium as $Cd^{2+}$, may gain access to biological membranes using transport pathways for essential metals, either by active or passive transport, such as the $Ca^{2+}$ and $Zn^{2+}$ transport channels or the $Fe^{2+}/H^{+}$ co-transporter divalent metal transporter 1 (Thevenod, 2010). The initial defence mechanism of cells is

through the induction of metallothioneins (MTs). MTs are low-molecular weight heat-stable high-cysteine content proteins that bind and transport cadmium within and across cells (Nordberg, 2009). In vertebrates, cadmium is transported to the liver where it binds to MTs and it is either redistributed to the rest of the organs, mainly the kidney and the testes (Keating et al., 2007; Migliarini et al., 2005), through the plasma or eliminated in the urine (Thompson and Bannigan, 2008).

Cadmium has been classified as a human carcinogen of category 1 and teratogen with probable mutagen properties (IARC 1993). It provokes genotoxicity to organisms, such as DNA strand breaks, chromosomal aberrations and micronuclei formations (MN) (Sarkar et al., 2015) through the indirect induction of reactive oxygen species (ROS) (Amirthalingam et al., 2013). Cadmium affects genome stability responsible for the cell's correct function and DNA replication and repair that may result in cell death through apoptosis (Bertin and Averbeck, 2006). It causes genotoxicity at concentrations that may indicate no apparent toxic effect to the organism (Pavlaki et al., 2016), which can eventually build up and generate harmful results to next generations and ultimately promote an impact at population level (Dixon et al., 2002). Taking into consideration these scenarios, relevant endpoints must be selected when performing this type of studies, in order to prevent individuals, and subsequently populations to be negatively impacted from any potential deleterious effects promoted by cadmium. Recent research has pointed out the potential that the single cell gel electrophoresis assay, also known as the comet assay, can have when combined with classic ecotoxicological endpoints (Chang et al., 2009; Coughlan et al., 2002; Depledge, 1998; Mai et al., 2012; Pavlaki et al., 2016; Sarkar et al., 2015). This methodology is simple, sensitive and fast to assess DNA damage and repair, representing therefore a reliable assessment tool in ecotoxicological studies (Frenzilli et al., 2009; Migliarini et al., 2005; Nigro et al., 2002; Sarkar et al., 2015).

## 1.4. Bioaccumulation studies and Toxicokinetic models

The assessment of a toxic effect initially attempts to cover the process leading from nominal to free metal ions concentrations and from that point to an adverse outcome in the organism. Besides the classic endpoints commonly used in ecotoxicology and mentioned in the previous section, bioaccumulation studies are usually used to describe the level of concentration of a contaminant in relation to the external surrounding concentration and may allow the estimation of possible risks at chemical concentration levels that are somewhat taken as moderate or controlled environmental concentrations.

When metal concentrations bioaccumulate in the organism and attain a threshold level, adverse effects can be observed in individuals or even at population level (Vijver et al., 2004). Bioaccumulation studies with the use of toxicokinetic models allow researchers to gain an additional insight in the way a contaminant, in the present study cadmium, can be absorbed from the surrounding environment and excreted by the target organism, using free metal ion activities to internal concentrations, and predicting how can those concentrations be associated to the effects it may promote over time (Directorate-General for Health and Food Safety and European Commission, 2013). The most common terms used in bioaccumulation studies are i) bioconcentration, ii) bioaccumulation and iii) biomagnification as given by Arnot and Gobas (2006):

i) Bioconcentration is the term used to describe the process where the organism uptakes the bioavailable form of metal solely through the surrounding water via respiratory and/or dermal pathways and the degree to which bioconcentration takes place is expressed using the bioconcentration factor (BCF) (**Figure 1.1**). BCFs can be calculated either as the ratio of the metal concentration measured in the organism at steady state (when the organism reaches a plateau, where the metal concentration in the organism does not vary in time and there is an equilibrium between uptake and excretion) by the bioavailable metal concentration in the surrounding media, or in case steady state is not achieved then the ratio of the kinetics parameters is estimated by the toxicokinetic model (TK) (described below) employed (OECD, 2012).

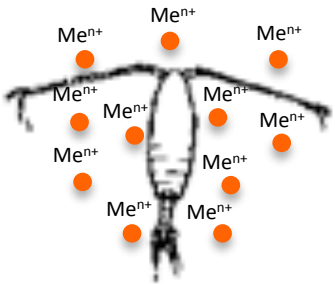


**Figure 1.1** Schematic representation of the bioconcentration process. A marine copepod is exposed to a metal ($Me^{n+}$) through the medium only.

ii) Bioaccumulation is the net concentration of a toxic substance in an organism by every possible pathway in the natural environment, e.g water, air, diet, soil or sediment (**Figure 1.2**). Just like the BCF, a bioaccumulation factor (BAF) is used to express the degree at which bioaccumulation occurs. As the BAF is mainly used to assess bioaccumulation under field conditions, it is estimated by the ratio of the metal concentration in the

organism at steady state and by the total metal concentration in the environment (Arnot and Gobas, 2006).

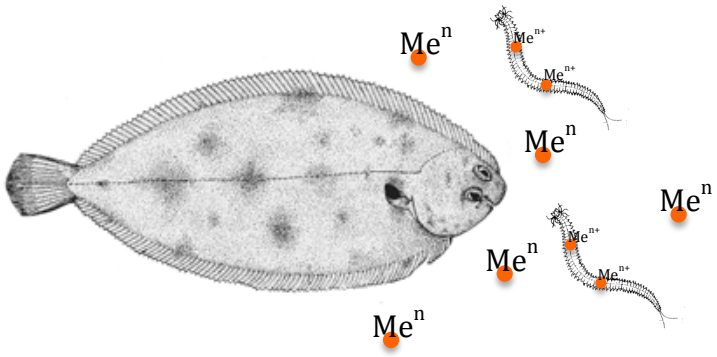


**Figure 1.2** Schematic representation of the bioaccumulation process. A marine flatfish is exposed to a metal ($Me^{n+}$) through the medium and through the diet.

iii) Biomagnification is the process to which metal concentration in the organisms is exceeding the metal concentration in the diet supplied along trophic levels (Arnot and Gobas, 2006) (**Figure 1.3**). Biomagnification factors (BMF) can be calculated either as the ratio of the metal concentration measured in the organism at steady state by the bioavailable metal concentration in the contaminated diet, or if steady state has not been achieved, the ratio of the kinetics parameter estimated by toxicokinetic models (TK) employed, as explained above (OECD, 2012).

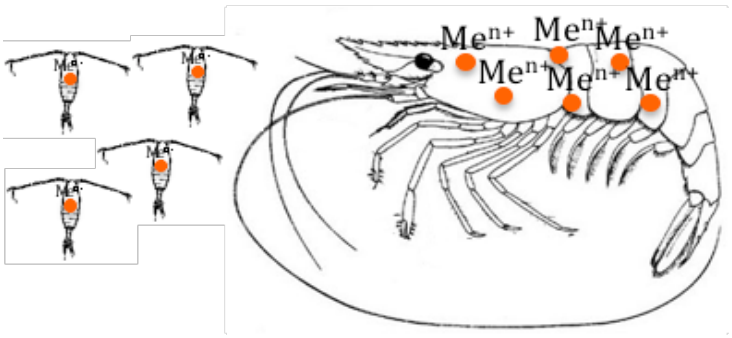


**Figure 1.3** Schematic representation of the biomagnification process. An estuarine shrimp showing higher leves of metal ($Me^{n+}$) compared to the concentration in its diet (marine copepods/lower trophic level).

For the prediction of bioaccumulation effects in the organisms, uptake and elimination kinetic studies are useful. Toxicokinetic models (TK) have been developed and employed

in the last years, as their outcome provides a better understanding of the internal body concentration, the uptake and elimination rates in the organism, bioconcentration/bioaccumulation/biomangification factors and the half-life of the target chemical. This is carried out by simulating short-term exposure conditions that may be extrapolated to assisting risk assessment management and regulatory procedures for the environment and subsequently for humans (Ashauer and Escher, 2010; Directorate-General for Health and Food Safety and European Commission, 2013; Dorne and Renwick, 2005).

In a classic one-compartment toxicokinetic study, the organisms are seen as one compartment where the influx and outflow of a chemical is studied, by assessing exposure to non-toxic concentrations during an uptake phase, followed by a depuration phase in contaminant-free medium. Body concentration of the chemical is measured in the test organisms at several points in time and toxicokinetic models are fit to the data. The kinetics parameters of chemicals, such as uptake and depuration rate constants, can be obtained from these models. In this way, not only can toxicokinetic studies provide valuable information for risk assessment (Ashauer and Escher, 2010) but it can also be very useful for regulatory purposes (Arnot and Gobas, 2006). Generally, a first-order one-compartment toxicokinetic model is considered when one needs to estimate metal uptake and depuration from the organism (Ardestani et al., 2014). Kinetics parameters are estimated with the use of **Equation 1.1** and **1.2** as described below:

For the uptake phase:

$Q(t) = Co + \frac{k_1}{k_2} * C_e * (1 - e^{(-k_2 * t)})$ (**Eq. 1.1**), for 0 ≤ t ≤ tc

For the depuration phase:

$Q(t) = Co + \frac{k_1}{k_2} * C_e * (e^{(-k_2 * (t - t_c))} - e^{(-k_2 * t)})$ (**Eq. 1.2**), for t > tc

where Q(t) is the internal concentration in the organism at the sampling time t, $C_0$ is the background concentration in the organism at time 0, $k_1$ is the uptake rate constant either from the water or from the diet, $k_2$ is the depuration rate constant, $C_e$ is the exposure concentration in the water or in the diet, $t_c$ is the time when the organisms where transferred to fresh uncontaminated medium and t is the sampling time.

Several versions of the model can be used according to the variables. The model equations may vary when i) the background concentration $C_0$ is involved (Ardestani et al., 2014; Janssen et al., 1991):

1. $C_0$=0, where no background concentration is present in the organisms (**Figure 1.4(1)**).

The models for uptake (**Equation 1.3**) and depuration (**Equation 1.4**) phase then read as:

$Q(t) = \frac{k_1}{k_2} * C_e * (1 - e^{(-k_2 * t)})$ (**Eq. 1.3**), for $0 \leq t \leq t_c$

$Q(t) = \frac{k_1}{k_2} * C_e * (e^{(-k_2 * (t - t_c))} - e^{(-k_2 * t)})$ (**Eq. 1.4**), for $t > t_c$

2. $C_0$ is a measured concentration at t=0 but cannot be eliminated from the organism, then the same equations as **1.1** and **1.2** are used and the background concentration in the organism can either be a fixed value or allowed to be estimated along the other kinetics parameters (**Figure 1.4(2)**).

or ii) when the bioavailable metal is absorbed and stored as an inert fraction in the organism and does not get eliminated in time (Tourinho et al., 2015; Vijver et al., 2006). Kinetics parameters can then be estimated using **Equation 1.1** for the uptake phase while for the depuration phase, **Equation 1.7** describes the existence of a Fi inert fraction in the organism (**Figure 1.4(3)**).

$Q(t) = Co + \frac{k_1}{k_2} * C_e * (Fi + (1 - Fi) * e^{(-k_2 * (t - t_c))})$ (**Eq. 1.7**), for $t > t_c$

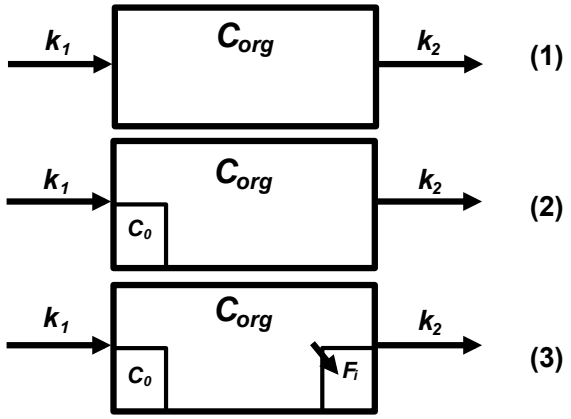


**Figure 1.4** Possible bioaccumulation schematic processes of metals in an organism. $C_{org}$ is the internal concentration in the organism at the sampling time t, $C_0$ is the background concentration in the organism at time 0, $k_1$ is the uptake rate constant either from the water or from the diet, $k_2$ is the depuration rate constant and Fi is the metal stored in the organism as an inert fraction (adapted from Ardestani et al., 2014)

As will be described further, in the work presented in this thesis, the abovementioned versions of the first-order one-compartment toxicokinetic model were used to assess, describe and predict uptake and elimination of cadmium in a time course with the use of three marine model species. The TK models aim at describing the internal fate of

cadmium (stored or eliminated) and explain internal accumulated concentrations though uptake and depuration processes.

## 1.5. Test Organisms

### 1.5.1. Invertebrates

*Acartia tonsa*

Zooplankton plays an important role in the trophic chains of marine and brackish ecosystems, as they are a main component of the dietary regimes of several fish and macro-invertebrates and a strong link for trace element reallocation between trophic levels (Fisher et al., 2000; Gissi et al., 2013; Rasdi and Qin, 2016). Copepods are one of the predominant zooplankton groups in marine ecosystems, being widely distributed and often the dominant group in zooplankton blooms (Xu et al., 2001).Their use in aquaculture as a live feed has increased over the last years due to its superior nutritional profile (high contents of highly unsaturated fatty acids) compared to the commonly used *Artemia* and/or rotifers (Ajiboye et al., 2010; Rasdi and Qin, 2016). They are usually used in ecotoxicological studies as they are considered sensitive indicators of metal pollution (Bao et al., 2013; Barka et al., 2010; Moraïtou-Apostolopoulou et al., 1979; Pedroso et al., 2007; Toudal and Riisgard, 1987; Xu et al., 2001).

Among marine copepods, *Acartia tonsa* Dana, 1849 (**Figure 1.5**) has been widely used in ecotoxicological tests and standardized protocols already exist for this micro-crustacean, namely the ISO 14669 (ISO, 1999) guideline for the determination of acute lethal toxicity to marine copepods, and the ISO 16778 (ISO, 2015) guideline for the determination of toxic effects on its early life stages. It is a euryhaline and eurythermic planktonic calanoid copepod with a worldwide distribution and commonly found in European coast waters (Leandro et al., 2006). After hatching, *A. tonsa* undergo several developmental stages, namely six naupliar and six copepodites, with the last copepodite stage being the adult one. The rationale supporting the use of *A. tonsa* as model species in the present work was as follows: i) it is simple and easy to maintain under controlled laboratory conditions, ii) it displays a short life-cycle (3 weeks from egg stage to adult) and a suitable reproductive potential (a female *A. tonsa* may deposit up to 60 eggs per day), iii) it is an important link between primary producers and predators in higher trophic levels and iv) it is sensitive to a variety of contaminants, such as metals or pesticides.

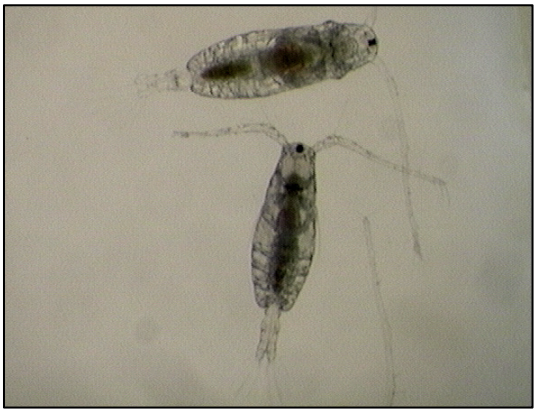

**Figure 1.5** *Acartia tonsa* adults. Female copepods are slightly bigger with longer and straighter antennae (top), while male copepods show antennae slightly smaller and curved (centre) (source: ISO, 2015).

*Palaemon varians*

The Atlantic ditch shrimp *Palaemon varians* Leach, 1813 (formerly known as *Palaemonetes varians*) (**Figure 1.6**) is a decapod crustacean within the Infraorder Caridea and a member of the Family Palaemonidae. It is a eurythermic and euryhaline species with a wide geographic distribution in Europe, commonly being recorded in shallow waters, namely in salt marshes and estuaries (Morris et al., 2015). *P. varians* is a detritivorous organism that also feeds on zooplankton, such as the larval stages of insects, polychaetes, and copepods. Decapod crustaceans, like *P. varians*, are meroplanctonic organisms, where their life cycle has two distinct phases: their larval stages are planctonic and after settlement their postlarval stages assume the benthic/demersal lifestyle of adult organisms.

Along the years, several studies have pointed out the importance of evaluating the accumulation potential and possible trophic transfer of several metals in estuarine shrimps using different pathways (water or prey) (Boisson et al., 2003; Rainbow et al., 2006a; 2006b; Rainbow and Smith, 2010; Seebaugh et al., 2006; Wallace et al., 2000; 1998). When exposed to different metal concentrations *P. varians* is either able to regulate essential metals (e.g. zinc) up to a certain level (Nugegoda and Rainbow, 1989) or store and subsequently detoxify non-essential metals (e.g. cadmium) (Rainbow and White, 1989). It is long known that decapod shrimp have the ability of accumulating high levels of metals, mainly in their midgut gland (Berillis et al., 2013; Chiodi-Boudet et al., 2013;

Gonçalves et al., 1989; Howard and Hacker, 1990), as this is the main organ for storage, detoxification and binding proteins in this group of organisms (e.g. metallothioneins (MTs)) (Chang et al., 2009; Kaoud and Eldahshan, 2010), due to the existence of different types of cells responsible for those processes (Correia et al., 2002). Briefly, the midgut gland is characterized by four cell types: the E- or embryonic from which all cells originate, the R- or resorptive and F- or fibrillar, with both being responsible for assimilation, storage and transportation of nutrients and metals, and the B- or blister cells, responsible for secretion, storage and transport to the intestine (Berillis et al., 2013; Ceccaldi, 1989). Previous authors have suggested decapod crustaceans as bioindicators of water conditions due to its midgut gland acting as a storage compartment for several contaminants and the possible induction of structural changes down to a cellular level by environmental stress, e.g. metal or organic contamination (Collins, 2010; Correia et al., 2002; Keating et al., 2007) and to its high sensitivity to metallic or organic compounds when compared with several estuarine fish (Key et al., 2006).

*P. varians* is of great economic importance as it is destined for human consumption or used as a live prey in fish aquaculture (Palma et al., 2008). Its use in fisheries and the fact that it is considered an important food resource for ichthyofauna, such as sea bass, eel and cuttlefish (Laffaille et al., 2001; Sykes et al., 2006), makes it an important link in the potential transfer of nutrients and metals to higher trophic levels in marine and brackish ecosystems (e.g., fish, birds and humans) (Seebaugh et al., 2006; 2005), hence holding the potential to promote a bottom-up transfer of hazardous chemicals.

**Figure 1.6** *Palaemon varians* adult specimen under culture conditions in laboratory (source: unknown).

### 1.5.2. Vertebrates

*Solea senegalensis*

The Senegalese sole (*Solea senegalensis*, Kaup, 1858) (**Figure 1.7**) is a marine demersal pleuronectid teleost fish with great economic value, as it is one of the most abundant flatfish species along the Atlantic and Mediterranean coasts (Bejarano-Escobar et al., 2010). This species is found in sandy or muddy substrates and mainly feeds on benthic invertebrates, e.g. small crustacean and polychaetes (Cabral, 2000). Owing to its high commercial value, several field and laboratory studies have been carried out throughout the years for a better understanding and assessment of the effects that possible contaminants may have in soles (Costa et al., 2008; 2013; Fonseca et al., 2011; Kalman et al., 2010; Oliva et al., 2009; Solé et al., 2004; Vázquez et al., 1994), as well as to improve aquaculture techniques (Dinis, 1992; Dinis et al., 2000; 1999; Imsland et al., 2003).

Estuarine ecosystems are commonly colonised by *S. senegalensis*, which makes soles highly susceptible to metal exposure due to fluctuations in water quality, often a consequence of anthropogenic activities (Creighton and Twining, 2010; Goetze et al., 2014). Fish uptake and accumulate metals directly from surrounding water, through the gills and skin, and/or indirectly from their diet; they tend to accumulate metals uptake from the environment or their prey mainly in the liver or intestine (Jakimska et al., 2011a). Therefore, experiments using estuarine benthic fish, such as *S. senegalensis*, can be particularly important as along with estuarine shrimp, they are often used as bioindicators in biomonitoring studies for water quality assessment due to a number of biological features (e.g. body size, long life cycle) and their high capacity to accumulate contaminants in their tissues (Barhoumi et al., 2009; Zhou et al., 2008).

**Figure 1.7** *Solea senegalensis* juveniles used in the present study during acclimation period.

## 1.6. Aim and outline of the thesis

This study aimed to investigate the eco- and genotoxicity of cadmium along with its bioaccumulation potential in three model species, representing three different levels of a marine trophic chain. This work addressed questions concerning the eco- and genotoxicity of cadmium in the early life stages of the three model species described in the Model Species Section, as well as bioaccumulation patterns taking into account the fluctuation of abiotic factors or different routes of exposure. The final aim of this work was to determine whether cadmium is being transferred along two different trophic pathways, one between invertebrates and another between invertebrates and vertebrates. The invertebrate trophic pathway consisted of a primary consumer, represented by the calanoid copepod *Acartia tonsa*, to a secondary consumer, represented by the estuarine shrimp *Palaemon varians*; the second trophic pathway between invertebrates and vertebrates was investigated using the marine teleost fish *Solea senegalensis* as a final consumer and top predator by exposing it to both contaminated water + diet (a marine polychaete was used as contaminated diet).

In order to attain the objectives of this study, the research was divided into 4 different tasks, which are reported in this thesis in **Chapters 2** to **5**.

In the current chapter, **Chapter 1**, a brief overview is provided addressing cadmium availability and speciation in the marine environment, potential ecotoxic and genotoxic effects, as well as the toxicokinetic models used for describing cadmium accumulation in the model species chosen.

In **Chapter 2** the toxic effects of cadmium are addressed in an individual and cellular level on the three model species. The questions aimed to be answered in this chapter where i) is cadmium toxicity life stage and species specific and ii) does cadmium cause genotoxicity to the tested species? In order to answer these questions, three different species and different life stages of each one of them were used in order to assess the lethal and sublethal effects of free ionic cadmium, as well as to evaluate its genotoxicity.

In **Chapter 3**, the bioconcentration potential of cadmium in the marine calanoid, *Acartia tonsa,* was evaluated under different environmental conditions, namely variable pH, salinity and temperature. Information from **Chapter 2** on the toxicity of cadmium was used to expose the copepods for the bioconcentration tests. The main objective of this chapter was to establish a relationship between bioavailability of cadmium and bioconcentration patterns by the copepod. A first-order one-compartment TK model was employed in order to estimate uptake and depuration rates, half-times ($DT_{50}$) and bioconcentration factors (BCF) for each condition tested.

In **Chapter 4**, an approach was made to evaluate the bioaccumulation patterns of cadmium in the estuarine ditch shrimp, *Palaemon varians*, under different exposure routes; i) waterborne, ii) dietary and iii) simultaneous exposure to both contaminated water and diet. Information on toxicity from **Chapter 2 and 3** was used in this chapter to expose *A. tonsa* to cadmium before supplying it as food to *P. varians*. The aim of this chapter was to evaluate the uptake and depuration rates of cadmium under different exposure routes and to identify which route would possibly represent a potential worst-case scenario for the bioaccumulation of cadmium in *P. varians*.

In **Chapter 5**, the accumulation and pattern distribution of cadmium in the different tissues of a top predator, the Senegalese sole, *Solea senegalensis*, was assessed after being simultaneously exposed to contaminated diet, the invertebrate ragworm *Hediste diversicolor* and contaminated water. Finally, an attempt was made to assess the potential risks and implications to human health associated with the consumption of the edible fraction of the sole.

Lastly, in **Chapter 6**, a summary and an integration and discussion of results from previous chapters is provided, along with future guidelines and suggestions for further research.

## References

Agency for Toxic Substances and Disease Registry (ATSDR), 2012. Toxicological profile for Cadmium, U.S. Department of Health and Human Services, Public Health Service. Atlanta, GA.

Ajiboye, O.O., Yakubu, A.F., Adams, T.E., Olaji, E.D., Nwogu, N.A., 2010. A review of the use of copepods in marine fish larviculture. Rev Fish Biol Fisheries 21, 225–246. doi:10.1007/s11160-010-9169-3

Ali, M., Taylor, A., 2010. The effect of salinity and temperature on the uptake of cadmium and zinc by the common blue mussel, *Mytilus edulis* with some notes on their survival. Mesopot. J. Mar. Sci. 25, 11–30.

Amirthalingam, T., Velusamy, G., Pandian, T.J., 2013. Cadmium-induced changes in mitotic index and genotoxicity on *Vigna unguiculata* (Linn.) Walp. Journal of Environmental Chemistry and Ecotoxicology 5, 57–62. doi:10.5897/JECE11.008

Ardestani, M.M., van Straalen, N.M., van Gestel, C.A.M., 2014. Uptake and elimination kinetics of metals in soil invertebrates: a review. Environ Pollut 193, 277–295. doi:10.1016/j.envpol.2014.06.026

Arnot, J.A., Gobas, F.A., 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. Environ. Rev. 14, 257–297. doi:10.1139/a06-005

Ashauer, R., Escher, B.I., 2010. Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. J Environ Monit 12, 2056–2061. doi:10.1039/c0em00234h

Bao, V.W.W., Leung, K.M.Y., Lui, G.C.S., Lam, M.H.W., 2013. Acute and chronic toxicities of Irgarol alone and in combination with copper to the marine copepod *Tigriopus japonicus*. Chemosphere 90, 1140–1148. doi:10.1016/j.chemosphere.2012.09.022

Barhoumi, S., Messaoudi, I., Deli, T., Saïd, K., Kerkeni, A., 2009. Cadmium bioaccumulation in three benthic fish species, *Salaria basilisca*, *Zosterisessor ophiocephalus* and *Solea vulgaris* collected from the Gulf of Gabes in Tunisia. J Environ Sci 21, 980–984. doi:10.1016/S1001-0742(08)62371-2

Barka, S., Pavillon, J.-F., Amiard-Triquet, C., 2010. Metal distributions in *Tigriopus brevicornis* (Crustacea, Copepoda) exposed to copper, zinc, nickel, cadmium, silver, and mercury, and implication for subsequent transfer in the food web. Environ. Toxicol. 25, 350–360. doi:10.1002/tox.20505

Barka, S., Pavillon, J.F., Amiard, J.C., 2001. Influence of different essential and non-

essential metals on MTLP levels in the Copepod *Tigriopus brevicornis*. Comp Biochem Physiol C 128, 479–493. doi:10.1016/S1532-0456(00)00198-8

Bejarano-Escobar, R., Blasco, M., DeGrip, W.J., Oyola Velasco, J.A., Martín Partido, G., Francisco Morcillo, J., 2010. Eye development and retinal differentiation in an altricial fish species, the senegalese sole (*Solea senegalensis*, Kaup 1858). Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 314B, 580–605. doi:10.1002/jez.b.21363

Berillis, P., Simon, C., Mente, E., Sofos, F., Karapanagiotidis, I.T., 2013. A novel image processing method to determine the nutritional condition of lobsters. Micron 45, 140–144. doi:10.1016/j.micron.2012.10.014

Bertin, G., Averbeck, D., 2006. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). Biochimie 88, 1549–1559. doi:10.1016/j.biochi.2006.10.001

Bjerregaard, P., Depledge, M.H., 1994. Cadmium accumulation in *Littorina littorea*, *Mytilus edulis* and *Carcinus maenas*: the influence of salinity and calcium ion concentrations. Mar Biol 119, 385–395. doi:10.1007/BF00347535

Boisson, F., Cotret, O., Teyssié, J.L., El-Baradeï, M., Fowler, S.W., 2003. Relative importance of dissolved and food pathways for lead contamination in shrimp. Marine Poll Bull 46, 1549–1557. doi:10.1016/S0025-326X(03)00316-3

Burke, J., Handy, R.D., Roast, S.D., 2003. Effect of low salinity on cadmium accumulation and calcium homeostasis in the shore crab (*Carcinus maenas*) at fixed free $Cd^{2+}$ concentrations. Environ Toxicol Chem 22, 2761–2767. doi:10.1897/02-410

Cabral, H.N., 2000. Comparative feeding ecology of sympatric *Solea solea* and *S. senegalensis*, within the nursery areas of the Tagus estuary, Portugal. Journal of Fish Biology 57, 1550–1562. doi:10.1111/j.1095-8649.2000.tb02231.x

Campbel, P., 1995. Interactions between trace metals and aquatic organisms : A critique of the Free-ion Activity Model. Metal Speciation and Bioavailability in Aquatic Systems.

Cao, L., Huang, W., Shan, X., Xiao, Z., Wang, Q., Dou, S., 2009. Cadmium toxicity to embryonic-larval development and survival in red sea bream *Pagrus major*. Ecotoxicol Environ Safety 72, 1966–1974. doi:10.1016/j.ecoenv.2009.06.002

Ceccaldi, H., 1989. Anatomy and physiology of digestive tract of Crustaceans Decapods reared in aquaculture. Advances in Tropical Aquaculture.

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2013a. Waterborne cadmium impacts immunocytotoxic and cytogenotoxic endpoints in green-lipped mussel, *Perna*

*canaliculus*. Aquat Toxicol 142, 283–293. doi:10.1016/j.aquatox.2013.09.002

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2013b. Biochemical biomarker responses of green-lipped mussel, *Perna canaliculus*, to acute and subchronic waterborne cadmium toxicity. Aquat Toxicol 140, 303–313. doi:10.1016/j.aquatox.2013.06.015

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2012. Impairment of green-lipped mussel (*Perna canaliculus*) physiology by waterborne cadmium: relationship to tissue bioaccumulation and effect of exposure duration. Aquat Toxicol 124-125, 114–124. doi:10.1016/j.aquatox.2012.07.013

Chang, M., Wang, W.-N., Wang, A.-L., Tian, T.-T., Wang, P., Zheng, Y., Liu, Y., 2009. Effects of cadmium on respiratory burst, intracellular $Ca^{2+}$ and DNA damage in the white shrimp *Litopenaeus vannamei*. Comp Biochem Physiol C 149, 581–586. doi:10.1016/j.cbpc.2008.12.011

Chiffoleau, J.-F., Cossa, D., Auger, D., Truquet, I., 1994. Trace metal distribution, partition and fluxes in the Seine estuary (France) in low discharge regime. Marine Chemistry 47, 145–158. doi:10.1016/0304-4203(94)90105-8

Chiodi-Boudet, L., Polizzi, P., Romero, M.B., Robles, A., Gerpe, M., 2013. Lethal and sublethal effects of cadmium in the white shrimp *Palaemonetes argentinus*: a comparison between populations from contaminated and reference sites. Ecotoxicol Environ Safety 89, 52–58. doi:10.1016/j.ecoenv.2012.11.008

Collins, P., 2010. Environmental stress upon hepatopancreatic cells of freshwater prawns (Decapoda: Caridea) from the floodplain of Paraná River. Natural Science 02, 748–759. doi:10.4236/ns.2010.27094

Correia, A.D., Pereira, A.L., Costa, M.H., Carrapiço, F., 2002. Functional anatomy of the midgut gland of *Gammarus locusta* (Crustacea : Amphipoda). J Mar Biol Assoc UK 82, 201–204. doi:10.1017/S0025315402005362

Costa, P.M., Caeiro, S., Costa, M.H., 2013. Multi-organ histological observations on juvenile Senegalese soles exposed to low concentrations of waterborne cadmium. Fish Physiol Biochem 39, 143–158. doi:10.1007/s10695-012-9686-1

Costa, P.M., Lobo, J., Caeiro, S., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., DelValls, T.Á., Costa, M.H., 2008. Genotoxic damage in *Solea senegalensis* exposed to sediments from the Sado Estuary (Portugal): effects of metallic and organic contaminants. Mutat. Res. 654, 29–37. doi:10.1016/j.mrgentox.2008.04.007

Coughlan, B.M., Hartl, M., O'Reilly, S.J., Sheehan, D., Morthersill, C., van Pelt, F., O'Halloran, J., O'Brien, N.M., 2002. Detecting genotoxicity using the Comet assay

following chronic exposure of Manila clam T*apes semidecussatus* to polluted estuarine sediments. Marine Poll Bull 44, 1359–1365. doi:10.1016/S0025-326X(02)00254-0

Creighton, N., Twining, J., 2010. Bioaccumulation from food and water of cadmium, selenium and zinc in an estuarine fish, *Ambassis jacksoniensis*. Marine Poll Bull 60, 1815–1821. doi:10.1016/j.marpolbul.2010.05.025

Depledge, M.H., 1998. The ecotoxicological significance of genotoxicity in marine invertebrates. Mutat. Res. 399, 109–122.

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ Toxicol Chem 20, 2383–2396. doi:10.1002/etc.5620201034/full

Dinis, M.T., 1992. Aspects of the potential of *Solea senegalensis* Kaup for aquaculture: larval rearing and weaning to an artificial diet. Aquacult Res 23, 515–520. doi:10.1111/j.1365-2109.1992.tb00795.x

Dinis, M.T., Ribeiro, L., Conceição, L., Aragão, C., 2000. Larvae digestion and new weaning experiments in *Solea senegalensis*. CIHEAM.

Dinis, M.T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. Aquaculture 176, 27–38. doi:10.1016/S0044-8486(99)00047-2

Directorate-General for Health and Food Safety, European Commission, E.C., 2013. Assessing the New Challenged for Risk Assessment. European Commission. doi:10.2772/37863

Dixon, D.R., Pruski, A.M., Dixon, L.R.J., Jha, A.N., 2002. Marine invertebrate eco-genotoxicology: a methodological overview. Mutagenesis 17, 495–507.

Dorne, J.L.C.M., Renwick, A.G., 2005. The refinement of uncertainty/safety factors in risk assessment by the incorporation of data on toxicokinetic variability in humans. Toxicol. Sci. 86, 20–26. doi:10.1093/toxsci/kfi160

EC, E.C., 2005. Commission Directive 2005/87/EC of 5 December 2005 Amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on Undesirable Substances in Animal Feed as Regards Lead, Fluorine and Cadmium.

Elinder, C.G., 1985. Cadmium: uses, occurrence and intake. Cadmium and Health: a toxicological and epidimiological appraisal, Florida.

Engel, D.W., Fowler, B.A., 1979. Factors influencing cadmium accumulation and its toxicity to marine organisms. Environ Health Perspect 28, 81–88.

Environment Programme, U.N., 2008. Draft final review of scientific information on

cadmium.

Fisher, N.S., Stupakoff, I., Sanudo-Wilhelmy, S., Wang, W.X., Teyssié, J.L., Fowler, S.W., Crusius, J., 2000. Trace metals in marine copepods: a field test of a bioaccumulation model coupled to laboratory uptake kinetics data. Mar. Ecol. Prog. Ser. 194, 211–218. doi:10.3354/meps194211

Fonseca, V.F., França, S., Serafim, A., Company, R., Lopes, B., Bebianno, M.J., Cabral, H.N., 2011. Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*. Aquat. Toxicol. 102, 216–227. doi:10.1016/j.aquatox.2011.01.018

Fonseca, V.F., Vasconcelos, R.P., Tanner, S.E., França, S., Serafim, A., Lopes, B., Company, R., Bebianno, M.J., Costa, M.J., Cabral, H.N., 2015. Habitat quality of estuarine nursery grounds: Integrating non-biological indicators and multilevel biological responses in *Solea senegalensis*. Ecol Indic 58, 335–345. doi:10.1016/j.ecolind.2015.05.064

Fowler, S.W., 1990. Critical review of selected heavy metal and chlorinated hydrocarbon concentrations in the marine environment. Mar Environ Res 29, 1–64. doi:10.1016/0141-1136(90)90027-L

Frazier, J.M., 1979. Bioaccumulation of cadmium in marine organisms. Environ Health Perspect 28, 75–79.

Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The Comet assay for the evaluation of genotoxic impact in aquatic environments. Mutation Research/Reviews in Mutation Research 681, 80–92. doi:10.1016/j.mrrev.2008.03.001

GESAMP, (.J.G.O.E.O.T.S.A.O.M.P., 1991. Reducing Environmental Impacts of Coastal Aquaculture, GESAMP Report and Studies. Rome.

Gissi, F., Binet, M.T., Adams, M.S., 2013. Acute toxicity testing with the tropical marine copepod *Acartia sinjiensis*: optimisation and application. Ecotoxicol Environ Safety 97, 86–93. doi:10.1016/j.ecoenv.2013.07.008

Goetze, S., Matoo, O.B., Beniash, E., Saborowski, R., Sokolova, I.M., 2014. Interactive effects of $CO_2$ and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. Aquat Toxicol 149, 65–82. doi:10.1016/j.aquatox.2014.01.027

Gonçalves, M., Vilhena, M., Machado, L., Pescada, C., Demoura, M.L., 1989. Effect of Speciation on Uptake and Toxicity of Cadmium to Shrimp *Crangon-Crangon* (L.). Bull Environ Contam Toxicol 43, 287–294.

Goswami, P., Thirunavukkarasu, S., Godhantaraman, N., Munuswamy, N., 2014.

Monitoring of genotoxicity in marine zooplankton induced by toxic metals in Ennore estuary, Southeast coast of India. Marine Poll Bull 88, 70–80. doi:10.1016/j.marpolbul.2014.09.025

Gustafsson, J.P., 2013. Visual MINTEQ ver. 3.0/3.1 [WWW Document]. Visual MINTEQ - A free equilibrium speciation model. URL http://vminteq.lwr.kth.se/ (accessed 9.9.15).

Henry, F., Filipuci, I., Billon, G., Courcot, L., Kerambrun, E., Amara, R., 2012. Metal concentrations, growth and condition indices in European juvenile flounder (*Platichthys flesus*) relative to sediment contamination levels in four Eastern English Channel estuaries. J Environ Monit 14, 3211–3219. doi:10.1039/c2em30765k

Howard, C.L., Hacker, C.S., 1990. Effects of Salinity, Temperature, and Cadmium on Cadmium-Binding Protein in the Grass Shrimp, *Palaemonetes pugio*. Arch Environ Contam Toxicol 19, 341–347. doi:10.1007/BF01054976

Imsland, A.K., Foss, A., Conceição, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P., White, P., 2003. A review of the culture potential of *Solea solea* and *S. senegalensis*. Rev Fish Biol Fisheries 13, 379–408. doi:10.1007/s11160-004-1632-6

International Agency for Research on Cancer, 1993. IARC. Cadmium and cadmium compounds. International Agency for Research on Cancer.

ISO, 2015. Water quality - Calanoid copepod early - life stage test with *Acartia tonsa*, International Organisation for Standardization.

ISO, 1999. Water quality - Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea), International Organisation for Standardization.

Jakimska, A., Konieczka, P., Skóra, K., 2011a. Bioaccumulation of Metals in Tissues of Marine Animals, Part II: Metal Concentrations in Animal Tissues. Pol. J. Environ. Stud 20, 5, 1127-1146.

Jakimska, A., Konieczka, P., Skóra, K., Namiesnik, J., 2011b. Bioaccumulation of metals in tissues of marine animals. Part I: The role and impact of heavy metals on organisms. Pol J Environ Stud 20, 1117–1125.

Janssen, C.R., De Schamphelaere, K., Heijerick, D., Muyssen, B., Lock, K., Bossuyt, B., Vangheluwe, M., Van Sprang, P., 2000. Uncertainties in the Environmental Risk Assessment of Metals. Hum Ecol Risk Assess 6, 1003–1018. doi:10.1080/10807030091124257

Janssen, M.P.M., Bruins, A., De Vries, T.H., Van Straalen, N.M., 1991. Comparison of cadmium kinetics in four soil arthropod species. Arch Environ Contam Toxicol 20, 305–312. doi:10.1007/BF01064395

Kalman, J., Riba, I., Angel DelValls, T., Blasco, J., 2010. Comparative toxicity of cadmium

in the commercial fish species *Sparus aurata* and *Solea senegalensis*. Ecotoxicol Environ Safety 73, 306–311. doi:10.1016/j.ecoenv.2009.10.013

Kaoud, H.A., Eldahshan, A.R., 2010. Bioaccumulation of cadmium in the freshwater prawn *Macrobrachium rosenbergii*. Nature and Science.

Keating, J., Delaney, M., Meehan-Meola, D., Warren, W., Alcivar, A., Alcivar-Warren, A., 2007. Histological findings, cadmium bioaccumulation, and isolation of expressed sequence tags (ESTs) in cadmium-exposed, specific pathogen-free shrimp, *Litopenaeus vannamei* postlarvae. J Shellfish Res 26, 1225–1237. doi:10.2983/0730-8000(2007)26[1225:HFCBAI]2.0.CO;2

Key, P.B., Wirth, E.F., Fulton, M.H., 2006. A Review of Grass Shrimp, *Palaemonetes* spp., as a Bioindicator of Anthropogenic Impacts. Environmental Bioindicators 1, 115–128. doi:10.1080/15555270600685115

Kirkelund, G.M., Ottosen, L.M., Villumsen, A., 2010. Investigations of Cu, Pb and Zn partitioning by sequential extraction in harbour sediments after electrodialytic remediation. Chemosphere 79, 997–1002. doi:10.1016/j.chemosphere.2010.03.015

Lacoue-Labarthe, T., Warnau, M., Oberhänsli, F., Teyssié, J.L., Koueta, N., Bustamante, P., 2008. Differential bioaccumulation behaviour of Ag and Cd during the early development of the cuttlefish *Sepia officinalis*. Aquat Toxicol 86, 437–446. doi:10.1016/j.aquatox.2007.12.005

Laffaille, P., Lefeuvre, J.C., Schricke, M.T., Feunteun, E., 2001. Feeding ecology of o-group sea bass, *Dicentrarchus labrax*, in salt marshes of Mont Saint Michel Bay (France). Estuaries 24, 116–125. doi:10.2307/1352818

Leandro, S.M., Tiselius, P., Queiroga, H., 2006. Growth and development of nauplii and copepodites of the estuarine copepod *Acartia tonsa* from southern Europe (Ria de Aveiro, Portugal) under saturating food conditions. Mar Biol 150, 121–129. doi:10.1007/s00227-006-0336-y

Luoma, S.N., 1996. The developing framework of marine ecotoxicology: Pollutants as a variable in marine ecosystems? J Exp Mar Biol Ecol 200, 29–55. doi:10.1016/S0022-0981(96)02679-2

Mai, H., Cachot, J., Brune, J., Geffard, O., Belles, A., Budzinski, H., Morin, B., 2012. Embryotoxic and genotoxic effects of heavy metals and pesticides on early life stages of Pacific oyster (*Crassostrea gigas*). Marine Poll Bull 64, 2663–2670. doi:10.1016/j.marpolbul.2012.10.009

Migliarini, B., Campisi, A.M., Maradonna, F., Truzzi, C., Annibaldi, A., Scarponi, G., Carnevali, O., 2005. Effects of cadmium exposure on testis apoptosis in the marine

teleost *Gobius niger*. Gen Comp Endocrinol 142, 241–247. doi:10.1016/j.ygcen.2004.12.012

Moraïtou-Apostolopoulou, M., Verriopoulos, G., Lentzou, P., 1979. Effects of sublethal concentrations of cadmium as possible indicators of cadmium pollution for two populations of *Acartia clausi* (Copepoda) living at two differently polluted areas. Bull Environ Contam Toxicol 23, 642–649. doi:10.1007/BF01770019

Morcillo, P., Esteban, M.A., Cuesta, A., 2016. Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. Chemosphere 144, 225–233. doi:10.1016/j.chemosphere.2015.08.020

Morel, F.M.M., 1983. Principles of Aquatic Chemistry. Wiley-Interscience.

Morris, J.P., Thatje, S., Ravaux, J., Shillito, B., Hauton, C., 2015. Characterising multi-level effects of acute pressure exposure on a shallow-water invertebrate: insights into the kinetics and hierarchy of the stress response. J Exp Biol 218, 2594–2602. doi:10.1242/jeb.125914

Mubiana, V.K., Blust, R., 2007. Effects of temperature on scope for growth and accumulation of Cd, Co, Cu and Pb by the marine bivalve *Mytilus edulis*. Mar Environ Res 63, 219–235. doi:10.1016/j.marenvres.2006.08.005

Newman, M.C., 2014. Fundamentals of Ecotoxicology. CRC Press.

Nigro, M., Frenzilli, G., Scarcelli, V., Gorbi, S., Regoli, F., 2002. Induction of DNA strand breakage and apoptosis in the eel *Anguilla anguilla*. Mar Environ Res 54, 517–520. doi:10.1016/S0141-1136(02)00178-2

Nordberg, G.F., 2009. Historical perspectives on cadmium toxicology. Toxicol Appl Pharmacol 238, 192–200. doi:10.1016/j.taap.2009.03.015

Nugegoda, D., Rainbow, P.S., 1989. Effects of Salinity Changes on Zinc Uptake and Regulation by the Decapod Crustaceans *Palaemon elegans* and *Palaemonetes varians*. Mar Ecol Prog Ser 51, 57–75. doi:10.3354/meps051057

OECD, 2012. OECD Guidelines for the Testing of Chemicals, Section 3: Degradation and Accumulation Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure. OECD Publishing. doi:10.1787/9789264185296-en

Oliva, M., Garrido, M.C., Sales Márquez, D., González de Canales, M.L., 2009. Sublethal and lethal toxicity in juvenile Senegal sole (*Solea senegalensis*) exposed to copper: a preliminary toxicity range-finding test. Exp Toxicol Pathol 61, 113–121. doi:10.1016/j.etp.2008.06.001

OSPAR Commission, 2004. Cadmium. OSPAR Commission, Paris.

Palma, J., Bureau, D.P., Andrade, J.P., 2008. Effects of binder type and binder addition

on the growth of juvenile Palaemonetes varians and Palaemon elegans (Crustacea : Palaemonidae). Aquaculture International 16, 427–436. doi:10.1007/s10499-007-9155-5

Panda, B.B., Panda, K.K., 2002. Genotoxicity and Mutagenicity of Metals in Plants, in: Physiology and Biochemistry of Metal Toxicity and Tolerance in Plants. Springer Netherlands, Dordrecht, pp. 395–414. doi:10.1007/978-94-017-2660-3_15

Paquin, P.R., Zoltay, V., Winfield, R.P., Wu, K.B., Mathew, R., Santore, R.C., Di Toro, D.M., 2002. Extension of the biotic ligand model of acute toxicity to a physiologically-based model of the survival time of rainbow trout (*Oncorhynchus mykiss*) exposed to silver. Comp Biochem Physiol C 133, 305–343.

Pavlaki, M.D., Araújo, M.J., Cardoso, D.N., Silva, A.R.R., Cruz, A., Mendo, S., Soares, A.M.V.M., Calado, R., Loureiro, S., 2016. Ecotoxicity and genotoxicity of cadmium in different marine trophic levels. Environ Pollut 215, 203–212. doi:10.1016/j.envpol.2016.05.010

Pedroso, M.S., Bersano, J.G.F., Bianchini, A., 2007. Acute silver toxicity in the euryhaline copepod *Acartia tonsa*: influence of salinity and food. Environ Toxicol Chem 26, 2158–2165. doi:10.1897/06-485R.1

Plette, A., Nederlof, M.M., Temminghoff, E., van Riemsdijk, W.H., 1999. Bioavailability of heavy metals in terrestrial and aquatic systems: A quantitative approach. Environ Toxicol Chem 18, 1882–1890.

Rainbow, P.S., Poirier, L., Smith, B.D., Brix, K.V., 2006a. Trophic transfer of trace metals: subcellular compartmentalization in a polychaete and assimilation by a decapod crustacean. Mar Ecol Prog Ser 308, 91-100.

Rainbow, P.S., Poirier, L., Smith, B.D., Brix, K.V., Luoma, S.N., 2006b. Trophic transfer of trace metals from the polychaete worm *Nereis diversicolor* to the polychaete *N. virens* and the decapod crustacean *Palaemonetes varians*. Mar Ecol Prog Ser 321, 167–181. doi:10.3354/meps321167

Rainbow, P.S., Smith, B.D., 2010. Trophic transfer of trace metals: Subcellular compartmentalisation in bivalve prey and comparative assimilation efficiencies of two invertebrate predators. J Exp Mar Biol Ecol 390, 143–148. doi:10.1016/j.jembe.2010.05.002

Rainbow, P.S., White, S.L., 1989. Comparative strategies of heavy metal accumulation by crustaceans: zinc, copper and cadmium in a decapod, an amphipod and a barnacle. Hydrobiologia 174, 245–262. doi:10.1007/BF00008164

Rasdi, N.W., Qin, J.G., 2016. Improvement of copepod nutritional quality as live food for

aquaculture: a review. Aquacult Res 47, 1–20. doi:10.1111/are.12471

Ray, S., 1984. Bioaccumulation of cadmium in marine organisms. Experientia 40, 14–23.

Roast, S.D., Widdows, J., Jones, M.B., 2001. Effects of salinity and chemical speciation on cadmium accumulation and toxicity to two mysid species. Environ Toxicol Chem 20, 1078–1084. doi:10.1002/etc.5620200519

Sarkar, A., Bhagat, J., Ingole, B.S., Rao, D.P., Markad, V.L., 2015. Genotoxicity of cadmium chloride in the marine gastropod *Nerita chamaeleon* using comet assay and alkaline unwinding assay. Environ. Toxicol. 30, 177–187. doi:10.1002/tox.21883

Seebaugh, D.R., Estephan, A., Wallace, W.G., 2006. Relationship Between Dietary Cadmium Absorption by Grass Shrimp (*Palaemonetes pugio*) and Trophically Available Cadmium in Amphipod (*Gammarus lawrencianus*) Prey. Bull Environ Contam Toxicol 76, 16–23. doi:10.1007/s00128-005-0884-8

Seebaugh, D.R., Goto, D., Wallace, W.G., 2005. Bioenhancement of cadmium transfer along a multi-level food chain. Mar Environ Res 59, 473–491. doi:10.1016/j.marenvres.2004.07.003

Solé, M., Potrykus, J., Fernández-Díaz, C., Blasco, J., 2004. Variations on stress defences and metallothionein levels in the Senegal sole, *Solea senegalensis*, during early larval stages. Fish Physiol Biochem 30, 57–66. doi:10.1007/s10695-004-6786-6

Sunda, W.G., Engel, D.W., Thuotte, R.M., 1978. Effect of Chemical Speciation on Toxicity of Cadmium to Grass Shrimp, *Palaemonetes pugio*: Importance of Free Cadmium Ion. Environ. Sci. Technol. 12, 409–413. doi:10.1021/es60140a003

Sykes, A.V., Domingues, P.M., Andrade, J.P., 2006. Effects of Using Live Grass Shrimp (*Palaemonetes varians*) as the only Source of Food for the Culture of Cuttlefish, *Sepia officinalis* (Linnaeus, 1758). Aquaculture International 14, 551–568. doi:10.1007/s10499-006-9054-1

Thevenod, F., 2010. Catch me if you can! Novel aspects of cadmium transport in mammalian cells. Biometals 23, 857–875. doi:10.1007/s10534-010-9309-1

Thompson, J., Bannigan, J., 2008. Cadmium: Toxic effects on the reproductive system and the embryo. Reprod Toxicol 25, 304–315. doi:10.1016/j.reprotox.2008.02.001

Toudal, K., Riisgard, H.U., 1987. Acute and Sublethal Effects of Cadmium on Ingestion, Egg-Production and Life-Cycle Development in the Copepod *Acartia tonsa*. Mar. Ecol. Prog. Ser. 37, 141–146. doi:10.3354/meps037141

Tourinho, P.S., van Gestel, C.A.M., Morgan, A.J., Kille, P., Svendsen, C., Jurkschat, K., Mosselmans, J.F.W., Soares, A.M.V.M., Loureiro, S., 2015. Toxicokinetics of Ag in the terrestrial isopod *Porcellionides pruinosus* exposed to Ag NPs and $AgNO_3$ via soil

and food. Ecotoxicology 1–12. doi:10.1007/s10646-015-1585-7

van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Phar 13, 57–149.

Vázquez, R., González, S., Rodriguez, A., Mourente, G., 1994. Biochemical composition and fatty acid content of fertilized eggs, yolk sac stage larvae and first-feeding larvae of the Senegal sole (Solea senegalensis Kaup). Aquaculture 119, 273–286. doi:10.1016/0044-8486(94)90182-1

Vicente-Martorell, J.J., Galindo-Riaño, M.D., García-Vargas, M., Granado-Castro, M.D., 2009. Bioavailability of heavy metals monitoring water, sediments and fish species from a polluted estuary. J Hazard Mater 162, 823–836. doi:10.1016/j.jhazmat.2008.05.106

Vijver, M.G., Van Gestel, C., Lanno, R.P., Van Straalen, N.M., Peijnenburg, W., 2004. Internal metal sequestration and its ecotoxicological relevance: A review. Environ. Sci. Technol. 38, 4705–4712. doi:10.1021/es040354g

Vijver, M.G., Vink, J.P.M., Jager, T., van Straalen, N.M., Wolterbeek, H.T., van Gestel, C.A.M., 2006. Kinetics of Zn and Cd accumulation in the isopod *Porcellio scaber* exposed to contaminated soil and/or food. Soil Biol Biochem 38, 1554–1563. doi:10.1016/j.soilbio.2005.11.006

Wallace, W.G., Brouwer, T.M.H., Brouwer, M., Lopez, G.R., 2000. Alterations in prey capture and induction of metallothioneins in grass shrimp fed cadmium‐contaminated prey. Environ Toxicol Chem 19, 962–971. doi:10.1002/etc.5620190425

Wallace, W.G., Lopez, G.R., Levinton, J.S., 1998. Cadmium resistance in an oligochaete and its effect on cadmium trophic transfer to an omnivorous shrimp. Mar. Ecol. Prog. Ser. 172, 225–237.

Ware, G.W., 1999. Reviews of Environmental Contamination and Toxicology. Springer Science & Business Media.

Wood, C.M., Farrell, A.P., Brauner, C.J., 2012. Homeostasis and Toxicology of Essential Metals. Academic Press.

World Health Organization, 2003. Cadmium Review (No. 1).

Xu, Y., Wang, W.-X., Hsieh, D.P.H., 2001. Influences of metal concentration in phytoplankton and seawater on metal assimilation and elimination in marine copepods. Environ Toxicol Chem 20, 1067–1077. doi:10.1002/etc.5620200518

Zhou, Q., Zhang, J., Fu, J., Shi, J., Jiang, G., 2008. Biomonitoring: An appealing tool for assessment of metal pollution in the aquatic ecosystem. Anal. Chim. Acta 606, 135–

150. doi:10.1016/j.aca.2007.11.018

Zirino, A., Yamamoto, S., 1972. A pH-dependent model for the chemical speciation of copper, zinc, cadmium and lead in seawater. Limnol Oceanogr, 17, 5, 661-671.

# Chapter 2

# Ecotoxicity and genotoxicity of cadmium in different marine trophic levels

Maria D. Pavlaki, Mário J. Araújo, Diogo N. Cardoso, Ana Rita R. Silva, Andreia Cruz, Sónia Mendo, Amadeu M.V.M. Soares, Ricardo Calado, Susana Loureiro

Environmental Pollution, 2016, 215: 203-212
doi:10.1016/j.envpol.2016.05.010

# Ecotoxicity and genotoxicity of cadmium in different marine trophic levels

## 2.1. Abstract

Cadmium ecotoxicity and genotoxicity was assessed in three representative species of different trophic levels of marine ecosystems - the calanoid copepod *Acartia tonsa*, the decapod shrimp, *Palaemon varians* and the pleuronectiform fish *Solea senegalensis*. Ecotoxicity endpoints assessed in this study were adult survival, hatching success and larval development ratio (LDR) for *A. tonsa*, survival of the first larval stage (zoea I) and post-larvae of *P. varians*, egg and larvae survival, as well as the presence of malformations in the larval stage of *S. senegalensis*. In vivo genotoxicity was assessed on adult *A. tonsa*, the larval and postlarval stage of *P. varians* and newly hatched larvae of *S. senegalensis* using the comet assay. Results showed that the highest sensitivity to cadmium is displayed by *A. tonsa*, with the most sensitive endpoint being the LDR of nauplii to copepodites. Sole eggs displayed the highest tolerance to cadmium compared to the other endpoints evaluated for all tested species. Recorded cadmium toxicity was (by increasing order): *S. senegalensis* eggs < *P. varians* post-larvae < *P. varians* zoea I < *S. senegalensis* larvae < *A. tonsa* eggs < *A. tonsa* LDR. DNA damage to all species exposed to cadmium increased with increasing concentrations. Overall, understanding cadmium chemical speciation is paramount to reliably evaluate the effects of this metal in marine ecosystems. Cadmium is genotoxic to all three species tested and therefore may differentially impact individuals and populations of marine taxa. As *A. tonsa* was the most sensitive species and occupies a lower trophic level, it is likely that cadmium contamination may trigger bottom-up cascading effects in marine trophic interactions.

Keywords: *Acartia tonsa*, *Palaemon varians*, *Solea senagalensis*, DNA damage, comet

## 2.2. Introduction

During the last 50 years, the environmental hazard of cadmium has been assessed in aquatic and soil ecosystems (Burger, 2008; Nordberg, 2009). This naturally occurring metal, is found both in water and soil/sediments at low concentrations due to natural processes, such as volcanic eruptions, natural crust erosion and also anthropogenic activities, such as mining and smelting (World Health Organization, 2003). Being a common by-product of zinc mining, cadmium often runoffs into aquatic systems (Environment Programme, 2008) and subsequently ends up in brackish and marine environments (Chiodi-Boudet et al., 2013). This non-essential metal to life forms, is commonly toxic even at relatively low concentrations and can cause adverse effects due to its high bioaccumulation tendency (Chandurvelan et al., 2013a; 2012). Reported concentrations of cadmium in the marine environment ranging from less than 5 ng $L^{-1}$ (World Health Organization, 2003) to an average of 40 ng $L^{-1}$ in unpolluted surface waters (Ray, 1984), while up to 250 ng $L^{-1}$ can be recorded in coastal areas of northern Europe (World Health Organization, 2003). This feature has been associated with riverine inputs and/or due to direct human impact (Elinder, 1985; World Health Organization, 2003).

The International Agency for Research on Cancer (1993) classifies cadmium as a human carcinogen and teratogen with probable mutagenic properties. Indeed, this metal can induce genotoxicity in organisms, such as DNA strand breaks, chromosomal aberrations and micronuclei formations (MN) (Sarkar et al., 2015). Cadmium contamination can trigger the production of reactive oxygen species (ROS) (Amirthalingam et al., 2013), which have been suggested to be the promoters of genotoxicity (Bertin and Averbeck, 2006). Nocuous effects of chemicals are usually foreshadowed by cellular and/or molecular shifts that sometimes may only be perceptible over time through the shaping of adult populations (Migliarini et al., 2005). Therefore, under these scenarios it may be too late to take any suitable measures to mitigate the negative impacts associated with chemical contamination. Recent research has pointed out the need of assessing the genotoxic effects of chemicals in aquatic organisms (Chang et al., 2009; Sarkar et al., 2015). The single cell gel electrophoresis assay, also known as comet assay, has proven to be a good assessment tool, as it is considered a simple, sensitive and fast way to assess DNA damage and repair (Frenzilli et al., 2009; Migliarini et al., 2005; Nigro et al., 2002; Sarkar et al., 2015; Thompson and Bannigan, 2008).

Cadmium's toxicity to aquatic organisms varies significantly and depends mainly on the concentration of its free ionic form, rather than the concentration of total dissolved

cadmium (Engel and Fowler, 1979; Sunda et al., 1978). Marine toxicology, in comparison to freshwater toxicology, remains "poorly" studied, mostly due to the complexity and constraints associated with the calculations of chemical speciation of contaminants in seawater (Hayes and Kruger, 2014; Leung et al., 2001). Cadmium contamination is no exception to this rule. One possible way to overcome the bottlenecks associated with speciation is to assess cadmium concentration through the use of chemical equilibrium speciation models. Various chemical equilibrium models, such as MINEQL+, Visual MINTEQ or MINTEQA2, allow the quantification and speciation of metals, in order to better evaluate ecotoxic effects and facilitate comparisons between studies.

In this way, in order to gain further knowledge on the effects of cadmium contamination in different marine trophic levels, the present study tested the following null hypotheses: a) cadmium toxicity is not life stage or species specific; and b) cadmium does cause genotoxicity to tested species. Three different species, representing different marine trophic levels, were used to assess lethal and sublethal effects of free ionic cadmium, including the comet assay to evaluate genotoxicity. In addition, different life stages were also used in order to infer any differences in their sensitivity to cadmium exposure. *Acartia tonsa*, a primary consumer, is a marine/estuarine copepod that is part of the diet of several macro invertebrates and fish. It is easily maintained in large cultures under laboratory conditions and it is widely used and accepted as an alternative to *Artemia* in aquacultures due to its higher nutritional value and fatty acids profile (Ajiboye et al., 2010; Shields et al., 1999; Støttrup, 2003; 2000; Støttrup et al., 1986; 1998). *Palaemon varians*, the Atlantic ditch shrimp or grass shrimp, a secondary consumer, is an edible estuarine shrimp. It plays a key role in these ecosystems, as it feeds on decaying matter and promotes nutrient recycling; it is also an important prey for juvenile and larval fish stages of commercial importance (Sykes et al., 2006). *Solea senegalensis* is a benthic flatfish often considered a top predator commonly found in abundance around European, Atlantic and Mediterranean coasts. It is of high economic value for fisheries and aquaculture (Bejarano-Escobar et al., 2010; Dinis, 1992; Dinis et al., 1999).

## 2.3. Materials and Methods

### 2.3.1. Test Organisms

Copepod Culture

Full life-cycle cultures of the marine calanoid copepod, *Acartia tonsa*, were kept in artificial seawater (ASW) prepared by mixing a commercially available salt mixture (Tropic Marin® Pro Reef salt; Tropic Marine, Wartenberg, Germany) with freshwater purified by a four-stage reverse osmosis unit (Aqua-win RO-6080). Cultures were started from eggs (kindly provided by the Escola Superior de Tecnologia do Mar, IPL, Peniche, Portugal) using 15-L poly(methyl methacrylate) (PMMA) cylindrical-conical tanks provided with constant aeration (3 bubbles/sec), at a density of ≈130 adults $L^{-1}$. Salinity was kept at 20 ±1, temperature at 20 ±1 ºC and photoperiod at 16 h light: 8 h dark. Newly hatched copepod nauplii were separated into 15 L PMMA tanks by filtering them using a 125 µm mesh and fed daily ad libitum with the cryptophyte, *Rhodomonas lens* CCMP 739 (minimum 2 x $10^7$ cells/mL). Tanks were cleaned daily to collect resting eggs, remove dead organisms, fecal pellets and excess of food. Total water renewal was performed once a week. Eggs of *A. tonsa* were stored at 4 ºC for starting new cultures whenever necessary.

Shrimp Culture

Ovigerous female ditch shrimp *Palaemon varians* were collected from the end of April till middle of October from a non-polluted salt marsh at Troncalhada, Aveiro, Portugal (40°38'40.1"N, 8°39'52.0"W) (Rodrigues et al., 2011). All specimens were stocked at a temperature of 20 ºC, salinity 35±1 and a photoperiod of 16 h light: 8 h dark in a recirculated maturation system described by Calado et al. (2007) until larval hatching. Newly hatched larvae (zoea I) were either used to run ecotoxicity trials (see below) or raised in a recirculated rearing system described by Calado et al. (2008) until they had reached their first post-larval stage (≈12 days after hatching). Larvae were fed daily ad libitum with newly hatched *Artemia* nauplii and decapsulated *Artemia* cysts until used for testing.

## Sole stock

Fertilized eggs of *Solea senegalensis* (less than 12 h) were kindly provided by Safiestela, S.A. (group Sea8), a commercial sole hatchery in Póvoa de Varzim, Portugal. Fertilized eggs were transferred to the laboratory using sealed plastic bags (1/3 seawater and 2/3 oxygen saturated atmosphere) under constant temperature (18 ºC). Upon arrival they were stocked under continuous aeration in 2-L flat bottom conical glass vials at salinity 35±1 and a temperature of 18±1 ºC for 2-3 h prior to testing. Eggs were chosen according

to their developmental stage, being selected for testing when shifting from the blastula to the gastrula stage.

### 2.3.2. Test Chemicals

The chemical compound used in the present study was cadmium chloride anhydrous (CAS No. 10108-64-2, Sigma-Aldrich, Germany). Stock solutions of 100 mg of Cd $L^{-1}$ and 10 g of Cd $L^{-1}$ were prepared with ultrapure water using a Millipore® Academic Milli-Q system. Tested concentrations were then accomplished through dilutions in artificial seawater (ASW) (see above for details) adjusting the salinity to each organism's culture conditions. To confirm the actual concentrations in the spiking process, dilutions were made in ultrapure water and acidified samples of the dilutions as well as acidified samples from the stock solutions used were sent for analysis to LCA (Central Laboratory of Analysis, University of Aveiro, Portugal). Chemical analysis was performed by Inductively Coupled Plasma Mass Spectrophotometry (ICP – MS) using a calibration curve made with seven standards obtained by successive dilutions of multi-element standard ICPMS-71A, from Inorganic Ventures, Virginia, USA. The method was verified using a Certified Reference Material (CRM), 1643e available from NIST. Quantification limits were 0.1 µg $L^{-1}$ and detection limits were 0.33 µg $L^{-1}$. As mentioned before, free ionic cadmium is considered to be the bioavailable chemical species, responsible for causing toxicity to the organisms. Therefore, the chemical equilibrium model Visual MINTEQ ver. 3.0/3.1 (Gustafsson, 2013) was used to estimate the metal speciation of cadmium in ASW medium using the concentration of all salt constituents (information supplied by the manufacturer) to obtain the concentration (in form of percentage) of free ionic cadmium under every condition (**Table S2.1-S2.3**). Within the Visual MINTEQ model, the pH and temperature were fixed at 7.9 for salinity 20±1, for the copepod *A. tonsa* and at 8.0 for salinity 35±1 for the ditch shrimp *P. varians* with a constant temperature of 20 ºC and 18 ºC for salinity 35±1 for the sole *S. senegalensis*, while the ionic strength of the solution was allowed to be estimated by the described model.

### 2.3.3. *Acartia tonsa* Acute Toxicity Test

To assess the toxic effects of cadmium in the mortality/immobilization of *A. tonsa*, the ISO 14669 standard for water quality was used (ISO, 1999). A preliminary test using a wide range of concentrations (0.01 mg of total Cd $L^{-1}$ to 100 mg of total Cd $L^{-1}$) was performed

to determine a definitive range (**Table S2.1**) and accurately estimate the $LC_{50}$. A total of five treatments, plus a negative control, were used for every test; five replicates per treatment, with five animals per replicate, were employed. Each replicate contained 40 mL of medium. Tests were maintained in a controlled chamber at 20 ºC, in a 16 h light: 8 h dark photoperiod, without feeding. The medium was not renewed during the whole span of the test (48 h). Mortality/immobilization was assessed as the number of immobilized copepods after a gentle agitation at 24 and 48 h of exposure. Values for salinity, pH, dissolved oxygen and temperature were recorded at the beginning and at the end of the test to check for validity criteria. There was no mortality on control organisms. A sensitivity test using the reference substance 3,5 – dichlorophenol was performed periodically to ensure that copepods were within expected sensitivity limits (ISO, 1999).

### 2.3.4. *Acartia tonsa* Early Life Stage Toxicity Test

The ISO/CD 16778 (ISO, 2013) draft for water quality was employed to evaluate the toxic effects of cadmium on the survival, hatching success and development of early life stage of *Acartia tonsa*. The concentrations used for the Early Life Stage (ELS) toxicity test ranged from 6.87 µg of total Cd $L^{-1}$ to 110 µg of total Cd $L^{-1}$, according to results previously obtained from the acute toxicity test (**Table S2.1**) (the $LC_{10}$ value of 110 µg of total Cd $L^{-1}$ estimated with Probit analysis from the acute toxicity test was used as a highest exposure concentration for the ELS test with a multiplying factor of 0.5). A total of five treatments plus a negative control (with six replicates per chemical treatment and twelve replicates per control, with 70 – 90 eggs of *A. tonsa* per replicate) were used. Each replicate contained 40 mL of medium. Four additional hatching controls were used in 80 mL of medium to ensure that 3 days after the test, hatching success had reached more than 80%.

At the end of the test, Lugol solution was added to the replicates and unhatched eggs, nauplii and copepodites were counted using a stereomicroscope for assessing the hatching success and larval development ratio (LDR), where (**Equation 2.1**):

$$\mathrm{LDR} = \frac{\mathrm{No.\,Copepodites}}{\mathrm{No.\,Copepodites} + \mathrm{No.\,Nauplii}} \qquad (\textbf{Eq. 2.1})$$

### 2.3.5. *Palaemon varians* Acute Toxicity Test

The EPA OPPTS 850.1035 Ecological Effects Guidelines for Mysid Acute Toxicity Testing (1996) was adapted in order to evaluate the toxic effects of cadmium to the mortality/immobilization of the organism during the initial larval life and post larval stage of *P. varians*. A preliminary test using a wide range of concentrations (0.01 to 100 mg of total Cd $L^{-1}$) was used to determine the final range of concentrations presented in **Table S2.2**, in order to obtain a more accurate $LC_{50}$ value. A total of five treatments, for the larval stage, and six treatments, for the post–larval stage, plus a negative control were used for every test (five replicates per treatment and five animals per replicate). Each replicate contained 100 mL of medium for zoea I and 250 mL for the post-larvae. Tests were performed in a controlled chamber at 20 ºC, with a 16 h light: 08h dark photoperiod, without feeding and with the medium being fully renewed after 48 h. Mortality/immobilization was assessed as the number of immobilized zoea I and post-larvae after a gentle agitation at 24, 48, 72 and 96 h. Values for salinity, pH, dissolved oxygen and temperature were obtained at the beginning and end of the test to validate the test. No mortality was recorded for control organisms.

### 2.3.6. *Solea senegalensis* Fish Embryo Toxicity Tests

The exposure of *S. senegalensis* embryos was performed using 24–well plates and six cadmium concentrations plus one control by adapting the Fish Embryo Toxicity Test (FET) Nr. 236 (OECD, 2013), to the sole species used in this study. Two types of toxicity tests were performed: 1) an acute toxicity test with embryos; and 2) a sub-lethal toxicity test to observe any malformations on embryos or newly hatched larvae. Concentrations of cadmium ranged from 0.5 to 12 mg of total Cd $L^{-1}$ for the embryo survival and hatching success, as well as the larval survival acute toxicity test, and from 6.25 to 100 µg of total Cd $L^{-1}$ for the larval survival and malformations sub-lethal test (**Table S2.3**). One embryo was added to each well with 2 mL of solution, with exposure lasting for 96 h with the renewal of the medium being performed every other day. Each 24 well plate contained four internal controls and five replicates in tetraplicate (total of 20 fertilized eggs per treatment) for the acute toxicity test and ten replicates of the test concentration in triplicates (total of 30 fertilized eggs per treatment) for the sub-lethal treatments.

### 2.3.7. Single Cell Gel Electrophoresis Assay (Comet assay)

The absence of any perceivable effects in the tests described above for *A. tonsa*, *P. varians* and *S. senegalensis* was used as rationale to select the cadmium concentrations to perform the comet assay. Cadmium concentrations of 6.87 µg $L^{-1}$, 27.5 µg $L^{-1}$ and 110 µg $L^{-1}$ were tested in tetraplicate (each replicate using ≈100 adult specimens) for *A. tonsa*. For the shrimp, five *P. varians* zoea I and five post-larvae were used in triplicate and three concentrations (0.3 mg $L^{-1}$, 0.6 mg $L^{-1}$ and 1.2 mg $L^{-1}$). For *S. senegalensis,* 30 fertilized eggs were used in triplicate in a total of four concentrations (1.56 µg $L^{-1}$, 3,12 µg $L^{-1}$, 6.25 µg $L^{-1}$ and 12.5 µg $L^{-1}$). A negative (ASW) and a positive control ($H_2O_2$) were used in all exposures.

All organisms were exposed to cadmium for 48 h after which they were sampled and placed in 600 µL of phosphate buffered saline (PBS) with 10% of dimethyl sulfoxide (DMSO) and 20 µM ethylene diamine tetra-acetic acid (EDTA), as described by Nogueira et al. (2006) and Silva et al. (2015). Tissues were mechanically disintegrated and then centrifuged at 200 x g for 10 min at 4 ºC. The supernatant was discarded and the precipitate was re-suspended. 10 µL of the suspension was mixed with low melting point agarose (0.5% at 37 ºC) and spread onto microscope glass slides (pre-coated with normal melting point agarose of 1%). The slides were placed on ice for 10 min, to solidify the agarose, immersed in cold lysing solution (10 mM Tris-HCl, 100 mM EDTA, 2.5 M NaCl, 10% DMSO and 2% Triton-X, pH 10) for 2 h at 4 ºC, in the dark. Slides were then removed and placed into the electrophoresis tank, containing buffer solution (10 M NaOH (pH 10) and 200 mM $Na_2EDTA$), for 15 min to denature and unwind DNA. Electrophoresis was run for 10 min at 300 mA (43 V). Slides were then neutralized by washing 3 times with 0.4 M Tris-HCl, pH 7.5 at 4 ºC. The slides were left to dry overnight in the dark after being dehydrated with absolute ethanol. Prior to visualization in a fluorescence microscope (Carl Zeiss Axio Scope A1 Fluorescent Microscopy) under a 400x magnification, the slides were stained with 100 µL of ethidium bromide (20 µg/mL). DNA damage was visually scored on a 0 to 4 scale as described by Duthie and Collins (1997).One hundred cells per slide were scored according to the following scale: Type 0, no DNA damage and type 4, extensive DNA damage.

### 2.3.8. Statistical Analysis

Cadmium concentrations promoting 50% mortality to copepods, shrimp and soles, as well as, 20% and 50% effect on larval development ratio, molting from copepod nauplii to copepodites, hatching success of copepod eggs and hatching of sole larvae, as well as larval deformities, were calculated using probit analysis (95% confidence intervals).

For the comet assay, the percentage of DNA damage was calculated according to the total score of 100 cells that ranged between 0 (all cells with no damage) and 400 (all cells with comet type 4). The total comet score was calculated in accordance with the method of Duthie and Collins (1997): (number of cells in type 0 × (type) 0) + (number of cells in type 1 × (type) 1) + (number of cells in type 2 × (type) 2) + (number of cells in type 3 × (type) 3) + (number of cells in type 4 × (type) 4). All treatments were compared to the control using a one-way analysis for variance (ANOVA) followed by a Dunnett's test ($p<0.05$). A two-way ANOVA was performed followed by a Sidak multiple comparison test ($p<0.05$) to compare differences of DNA damage between the comet assays performed for the two life stages of *P. varians* (zoea I and post-larvae).

All calculations were performed using the SPSS Statistics package version 20 while all figures were created using the Graphpad Prism 6 statistical pack (Graphpad Software, La Jolla, CA, USA).

## 2.4. Results

### 2.4.1. Chemical Analysis – Cadmium Speciation

The concentration of cadmium in the stock solutions, as well as in ultrapure water, showed a deviation of less than 10% from the initial nominal concentrations validating the spiking technique.

The equilibrium model determined the percentages of free ionic cadmium concentration in ASW according to initial total cadmium concentrations and environmental conditions (temperature, salinity and pH). Free ion percentages were estimated to be 8.7% at salinity 20±1 and temperature 20 ºC (for the copepod *A. tonsa*), 4.5% at salinity 35±1 and temperature 20 ºC (for the shrimp *P. varians*) and 4.6% at salinity 35±1 and temperature 18 ºC (for the sole *S. senegalensis*) of the total cadmium concentration.

The concentration of each chemical species of cadmium of the different treatments is shown in **Table S2.1-S2.3**.

**Table 2.1** Values of lethal concentrations of cadmium (expressed as μg $Cd^{2+}$ $L^{-1}$) to adult copepods, larvae and postlarvae of shrimps and hatching and survival of sole larvae, as well as effect on hatching success and inhibition of naupliar stages to molt to copepodite stages of *Acartia tonsa* and malformations of *Solea senegalensis* larvae. n.d. – not determined, 95% confidence intervals could not be calculated, * indicates data extrapolated using probit analysis.

***Acartia tonsa***

| Acute Toxicity Test | | ELS Toxicity Test | | | |
|---|---|---|---|---|---|
| | | LDR | | Hatching Success | |
| **24h $LC_{50}$** | 79.8 (69.2-94.2) | **$EC_{20}$** | 1.82 (1.08-9.78) | **$EC_{20}$** | 4.51 (3.77-5.67) |
| **48h $LC_{50}$** | 64.4 (53.6-78.8) | **$EC_{50}$** | 0.69 (0-1.70) | **$EC_{50}$** | 1.94 (1.36-2.51) |

***Palaemon varians***

| Acute Toxicity Test | | | |
|---|---|---|---|
| Zoea I | | Postlarvae | |
| **24h $LC_{50}$** | - | **24h $LC_{50}$** | - |
| **48h $LC_{50}$** | - | **48h $LC_{50}$** | 319.1 (251.7-427-9) |
| **72h $LC_{50}$** | 99.6 (87.5-115.7) | **72h $LC_{50}$** | 168 (144.2-200.9) |
| **96h $LC_{50}$** | 41.9 (36.8-47.1) | **96h $LC_{50}$** | 67.7 (57.5-80.8) |

***Solea senegalensis***

| Acute Toxicity Test | | | | Fish Embryo Toxicity Test | | | |
|---|---|---|---|---|---|---|---|
| Hatching | | Larvae Survival | | Larvae Survival | | Larvae Malformations | |
| **24h $LC_{50}$** | 291.4 (n.d.) | **24h $LC_{50}$** | 30.9 (0-69.9) | **24h $LC_{50}$** | 34.4* (n.d.) | **24h $EC_{20}$** | 7.2* (n.d.) |
| | | | | | | **24h $EC_{50}$** | 10.1* (n.d.) |
| | | | | **48h $LC_{50}$** | 5.1 (3.7-9.7) | **48h $EC_{20}$** | 1.9 (1.3-2.4) |
| | | | | | | **48h $EC_{50}$** | 3.1 (2.6-3.9) |
| | | | | **72h $LC_{50}$** | 2.5 (1.9-3.3) | **72h $EC_{20}$** | 3.1 (2.1-7.7) |
| | | **48h $LC_{50}$** | 4.9* (n.d.) | | | **72h $EC_{50}$** | 5.4* (3.6-15.5) |
| | | | | **96h $LC_{50}$** | 2.5 (1.9-3.3) | **96h $EC_{20}$** | 2.3 (n.d.) |
| | | | | | | **96h $EC_{50}$** | 5.9* (n.d.) |

### *2.4.2. Acartia tonsa*

The lethal concentrations recorded for the acute toxicity test (50% survival of exposed adult copepods after 24 and 48 h), as well as effective concentrations (20% and 50%) from the ELS toxicity test (with the 95% confidence intervals) are summarized in **Table 2.1**.

Results show an increase on mortality with time in the acute toxicity test. After 24 and 48 h, no copepod survival was recorded at the highest concentration of cadmium. One-way ANOVA showed the existence of significant differences (Dunnett's, $p<0.05$) between the control group and the three highest concentrations tested (44 µg $Cd^{2+}$ $L^{-1}$, 78 µg $Cd^{2+}$ $L^{-1}$ and 141 µg $Cd^{2+}$ $L^{-1}$). From the ELS test, larval development ratio, as well as hatching success, showed a decrease with increasing concentration of cadmium. For the two highest concentrations tested, an LDR of 0 was recorded, thus evidencing that no copepod nauplii was able to reach the copepodite stage (**Figure 2.1**). Hatching success in controls varied from 84.4% to 94.7%, while at the two highest concentrations (4.79 µg $Cd^{2+}$ $L^{-1}$ and 9.57 µg $Cd^{2+}$ $L^{-1}$) the mean percentage of hatching decreased to 20.3% and 3.7% respectively. When compared to the control, all concentrations presented significant differences (ANOVA, for LDR, $F(5,28)=109.9$, $p<0.001$, and for hatching success, $F(5,28)=126.6$, $p<0.001$) (**Figure 2.1**).

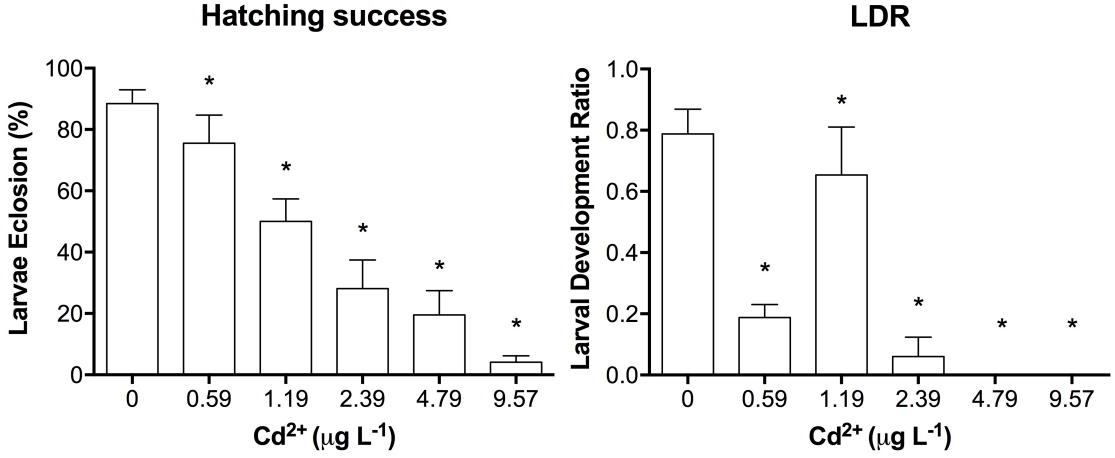


**Figure 2.1** Larval Development Ratio of *Acartia tonsa* nauplii to copepodids and Hatching Success of *Acartia tonsa* eggs after exposure to different concentrations of free $Cd^{2+}$ (µg $L^{-1}$) (* $p<0.05$, Dunnett's multiple comparison against the control).

### 2.4.3. Palaemon varians

The lethal concentrations of cadmium after 24, 48, 72 and 96 hours of exposed *P. varians* zoea I and post–larvae as well as the 95% confidence intervals are summarized in **Table 2.1**. After 24 h of exposure to the highest cadmium concentrations, no mortality was observed to zoea I larvae nor to post-larvae. After 48 h, the second highest concentration tested (217 µg $Cd^{2+}$ $L^{-1}$) did not affect survival of *P. varians* zoea I and post-larvae, while 100% mortality was recorded when the postlarval stages were exposed to the highest concentration of cadmium (434 µg $Cd^{2+}$ $L^{-1}$). After 72 h of cadmium exposure, larvae at zoea I displayed a mortality of 64% at 108 µg $Cd^{2+}$ $L^{-1}$, while post-larvae mortality, under that same concentration, was recorded to be less than 50%. After 96 h of exposure, at a concentration of 54 µg $Cd^{2+}$ $L^{-1}$, only 12% of zoea I survived, while post-larvae presented a higher survival (52%). As the concentration increased, survival of post-larvae decreased to 16% at 108 µg $Cd^{2+}$ $L^{-1}$. No survival was recorded at the two highest concentrations of cadmium, 108 µg $Cd^{2+}$ $L^{-1}$ and 217 µg $Cd^{2+}$ $L^{-1}$ and 217 µg $Cd^{2+}$ $L^{-1}$ and 434 µg $Cd^{2+}$ $L^{-1}$, for the larvae zoea I and the post – larvae, respectively.

### *2.4.4. Solea senegalensis*

Toxicity parameters on hatching success, survival of larvae and effects of cadmium on larval morphology along time are presented in **Table 2.1**. For both toxicity tests, hatching success and larval survival were significantly affected at all tested concentrations of cadmium. After 24 h of exposure no larval survival was observed at the two highest concentrations tested, 364 µg $Cd^{2+}$ $L^{-1}$ and 546 µg $Cd^{2+}$ $L^{-1}$, while after 48 h all concentrations in the acute test induced 100% mortality. Concerning the sub-lethal toxicity test, while all control eggs hatched after 48 h, the hatching success recorded in all treatments was not higher than 93%. Larval survival decreased as cadmium concentrations increased, with a similar trend being recorded from 24 to 48 hours (the percentage reduced to ~50%). After 72 and 96 h, the percentage of larvae surviving at 3.4 µg $Cd^{2+}$ $L^{-1}$ dropped to 17% and 13%, respectively, while at 4.6 µg $Cd^{2+}$ $L^{-1}$ it reached 10% and 6.7%, respectively (**Figure 2.2**). After 48 h of exposure, 48% and 53.6% of larvae presented malformations (such as tail and yolk sac deformities, as well as altered axial curvature (**Figure 2.3**)) at concentrations 3.4 and 4.6 µg $Cd^{2+}$ $L^{-1}$ (**Figure 2.2**).

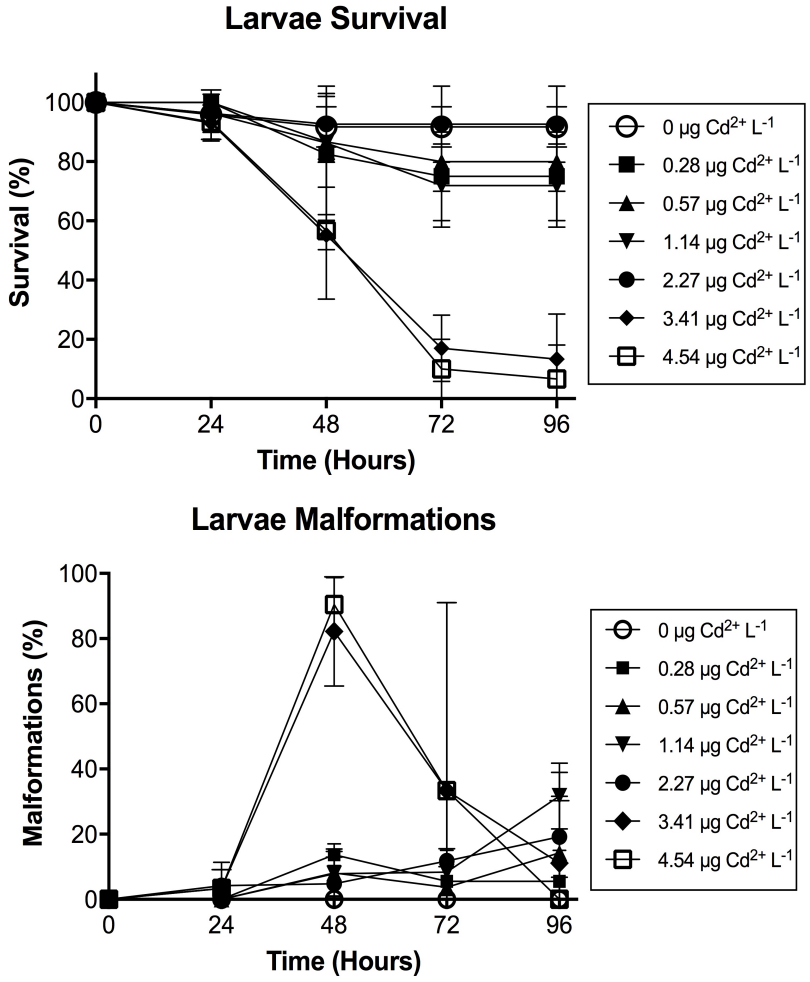


**Figure 2.2** Survival of *Solea senegalensis* larvae and malformations after exposure to different concentrations of free $Cd^{2+}$ (µg $L^{-1}$) over time.

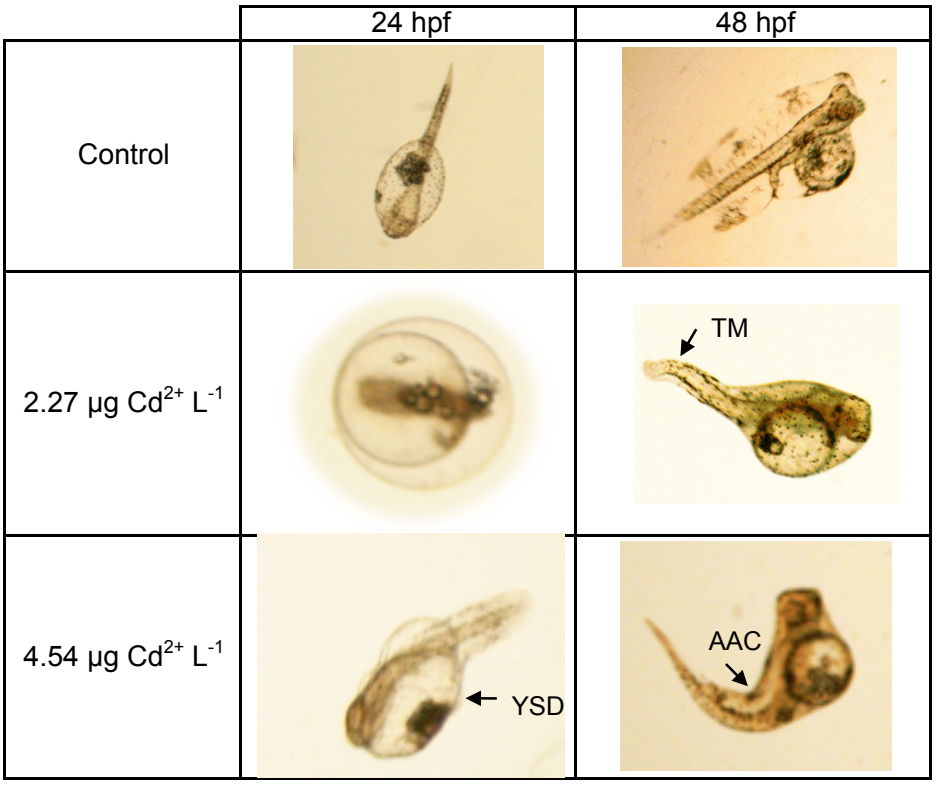


**Figure 2.3** Morphological malformations of *Solea senegalensis* embryos and larvae exposed to different concentrations of free $Cd^{2+}$. High $Cd^{2+}$ concentrations caused delay in larvae hatching after an exposure of 24 h, while tail malformations (TM), altered axial curvature (AAC) and yolk salk deformities (YSD) could be recorded after an exposure of 48 h.

### 2.4.5. Single Cell Gel Electrophoresis Assay (Comet assay)

Increasing cadmium concentrations induced an increase in DNA damage to all tested species. The percentage of damage recorded at all the concentrations was significantly higher (Dunnett's, $p<0.05$) in comparison to the control, for both *A. tonsa* (ANOVA, $F(3,28)=58.02$, $p<0.001$) and *P. varians* (ANOVA, $F(3,19)=40.61$, $p<0.001$ for zoea I and $F(3,20)=31.54$, $p<0.001$ for post-larvae). With respect to *S. senegalensis* larvae, the statistical analysis revealed significant differences in all the tested concentrations when compared to the control (ANOVA, $F(4,24)=510.8$, $p<0.001$), except for the lower concentration (**Figure 2.4**) where DNA damage was similar (DNA damage ± Std.Dev. = 32 ± 2.7%) to the control (DNA damage ± Std.Dev. = 29.4 ± 1.8%). No significant differences in DNA damage were recorded between *P. varians* zoea I and post-larvae exposed to the same concentrations of cadmium (two-way ANOVA, $F(1,39)=0.034$,

p=0.854). No mortality was recorded in any of the exposure trials; likewise, abnormalities were not observed in *S. senegalensis* embryos nor newly hatched larvae.

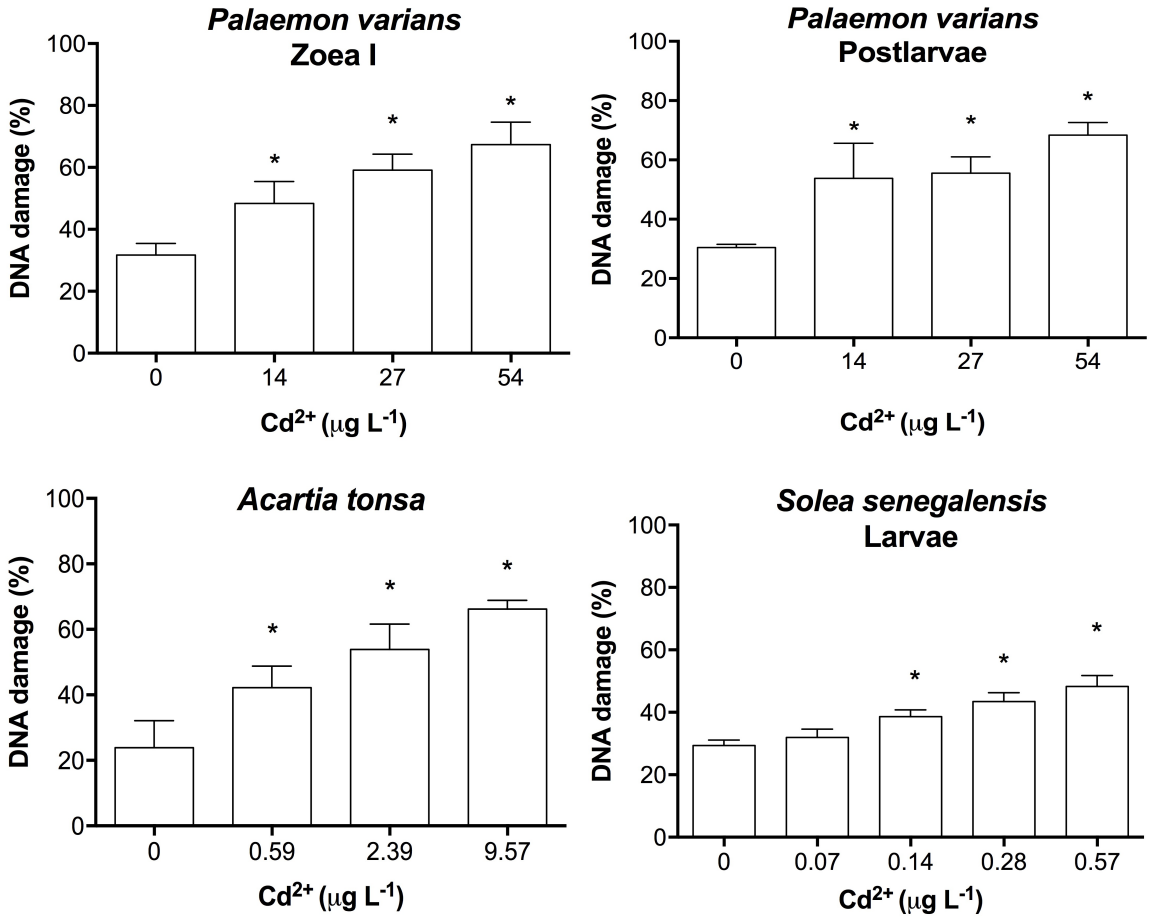


**Figure 4.** DNA damage of *Acartia tonsa*, *Palaemon varians* and *Solea senegalensis* cells exposed to different concentrations of free $Cd^{2+}$ (µg $L^{-1}$) after an exposure of 48 h (* $p<0.05$, Dunnett's multiple comparison against the control)

## 2.5. Discussion

### 2.5.1. Chemical Analysis – Cadmium Speciation

It has long been emphasized the need to gain an in depth knowledge on metal chemical speciation to evaluate and better understand how metals behave in the marine environment. Sunda et al. (1978) and Engel and Fowler (1979) early highlighted that free $Cd^{2+}$ species is generally the most toxic form of cadmium and is inversely correlated to

salinity. Zirino and Yamamoto (1972) showed the high affinity of cadmium to chloride ions. These authors determined that the percentage of ionic cadmium concentration to total cadmium concentration was ≈2.5% over a pH ranging from 7 up to 9, highlighting that the predominant species were cadmium chloride complexes (mainly $CdCl_2$ and $CdCl^-$). Subsequently, several other authors continued to address the issue of cadmium chemical speciation in seawater (**Table 2.2**).

**Table 2.2** Values of ionic cadmium (given in percentage) estimated by chemical equilibrium speciation equations/models in previous studies.

| Exposure Medium | Salinity | Temperature (ºC) | pH | Ionic Cadmium (%) | Reference |
|---|---|---|---|---|---|
| Natural Seawater | 35 | 25 | 8.2 | 3 | Turner et al. 1981 |
| Natural Seawater | 22 | 22.2 | - | 5.8 | De Lisle et al. 1988 |
| | 30 | 22.2 | - | 3.8 | |
| | 38 | 22.2 | - | 2.6 | |
| Artificial Seawater (commercial formula NaCl) | 33 | 10 | 6.8 | <7 | Rainbow et al. 1993 |
| Natural Seawater | 24 | 23 | - | 5.4 | De Lisle et al. 1994 |
| | 32 | 23 | - | 3.4 | |
| Artificial Seawater (Instant Ocean®) | 30 | 15 | - | 3.85 | Roast et al. 2001 |
| | 10 | 15 | - | 14.8 | |
| Natural Seawater | 10.5 | 14 | 7.96 | 13 | Burke et al. 2003 |
| | 32 | 14 | 7.96 | 3.4 | |
| Artificial Seawater (Tropic Marin® Pro Reef) | 20 | 20 | 7.9 | 8.7 | Present study |
| | 35 | 20 | 8.0 | 4.5 | |
| | 35 | 18 | 8.0 | 4.5 | |

In the present study the vMINTEQ speciation model estimated free ionic cadmium to be 4.5% of the total cadmium at a salinity of 35 and 8.7% at salinity of 20. Overall, data from the present study is in line with the previous studies summarized in **Table 2.2**. Minor differences in the percentages estimated could be due to different concentration of salt

components (e.g., higher concentration of chloride ions) used to prepare synthetic seawater. Indeed, different studies use different commercial brands of synthetic salts (e.g., Roast et al. (2001) used the brand Instant Ocean®, while the present study used Tropic Marin®), while others relay on commercial formulas of NaCl (Rainbow et al., 1993) or simply employ natural filtered seawater (Burke et al., 2003; De Lisle and Roberts, 1994; 1988; Turner et al., 1981).

### *2.5.2. Acartia tonsa*

Previous studies have already demonstrated the deleterious effects that cadmium can have at a cellular, individual and population level in marine systems. Results from the acute and early life stages tests show that the earlier life stages of copepods appear to be more sensitive to cadmium than adult conspecifics (**Table 2.1**). While a concentration of 1.94 µg $Cd^{2+}$ $L^{-1}$ already promoted a decrease in egg viability impairing the hatching of 50% of copepod nauplii, a 30-fold concentration increase (64.4 µg $Cd^{2+}$ $L^{-1}$) was necessary to decline the survival of adult copepods by 50% (**Table 2.1**). Moraitou – Apostolopoulou et al. (1979) reported an $LC_{50}$ value of 600 µg Cd $L^{-1}$ for the copepod *Acartia clausii*, a significantly higher value compared to the one reported in this study. Cadmium toxicity on a different copepod species, *Tisbe battagliai*, appears to be lower, as Hutchinson et al. (1994) reported an $LC_{50}$ value after 96 hours of 340 µg $Cd^{2+}$ $L^{-1}$ for adult specimens. Such differences may be related to the way that data is presented (total cadmium values *vs* free ionic cadmium), as well as differences on testing conditions: salinity of the test medium (e.g., natural seawater at salinity 35 *vs* synthetic seawater at salinity 20 in the present study); or different temperatures (e.g., 25 ºC vs. 20 ºC, present study). Verriopoulos and Moraitou – Apostolopoulou (1982) also reported that the most sensitive life stage of the copepod *Tisbe holothouridae* was the one-day-old nauplii, namely when compared to adults. Another study shows that the LDR parameter was the most sensitive for the copepod *Nitocra spinipes* exposed to four different synthetic musks (Breitholtz et al., 2003). Several studies attribute the high sensitivity of earlier life stages to toxicants to undeveloped detoxifying organs, thus displaying less effective homeostatic mechanisms to regulate metal ions transport (Mohammed, 2013).

### *2.5.3. Palaemon varians*

Cadmium has negatively affected the survival of both larval and postlarval stages of *P. varians* over time. After 96 h of cadmium exposure, zoea I of *P. varians* appear to be more sensitive than post-larvae, with an $LC_{50}$ of 41.9 µg $Cd^{2+}$ $L^{-1}$ compared to 67.7 µg $Cd^{2+}$ $L^{-1}$, respectively (**Table 2.1**). This result supports the idea that sensitivity to cadmium decreases along species ontogeny. Similar results were also reported by Bambang et al. (1995), as newly hatched larvae of the shrimp *Penaeus japonicus* were more sensitive to cadmium than either postlarval stages or juveniles (96 h $LC_{50}$=10-30 µg Cd $L^{-1}$ for zoea, $LC_{50}$=200-3500 µg Cd $L^{-1}$ for post-larval stages and $LC_{50}$=5500 µg Cd $L^{-1}$ for juveniles). Cripe (1994) showed that ≤24 h old mysid, *Mysidopsis bahia*, to be more sensitive (26-fold increase) to cadmium when compared to post larval pink shrimp, *Penaeus duorarum*. Moreover, Howard and Hacker (1990) defined an $LC_{50}$ value of 2.42 µg Cd $L^{-1}$ after 96 h of exposure for the adult stage of *Palaemonetes pugio* to cadmium. Verriopoulos and Moraitou – Apostolopoulou (1982) demonstrated that early ontogenic stages (e.g., larvae) regulate metal ion transport less effectively than latter ones (e.g., juveniles and adults), thus showing an increasing sensitivity to these compounds. These findings are in line with the data reported in our study.

### *2.5.4. Solea senegalensis*

Literature on the chemical toxicity of cadmium to several marine vertebrates shows that sensitivity can be species and developmental stage specific. Along their life cycle, these organisms can become more tolerant to these compounds although bioaccumulation is more likely to occur (Araujo et al., 2013; Cao et al., 2009; Mohammed, 2013). In this sense, hatchability and larval survival have been used as reliable and sensitive biological endpoints to account for chemical toxicity. Results from the present study show that larvae were more sensitive to cadmium than embryos (**Table 2.1**). As already highlighted by previous authors, it is legitimate to assume that at such an early stage, the existence of the chorionic membrane may act as an effective barrier between fish embryos and environmental toxicants (Eaton et al., 1978; Mance, 1987; Mhadhbi et al., 2010; Plhalová et al., 2011). Mhadhbi et al. (2010) reports a 50% decrease in the hatching success of embryos of the marine flatfish *Psetta maxima* (turbot) at a concentration of 112.3 µg of Cd $L^{-1}$, while Rombough and Garside (1982) reported that at concentrations higher than 270 µg $L^{-1}$ larval hatching decreased by more than 40% for *Salmo salar* (Atlantic salmon). Cao

et al. (2009) reports $LC_{50}$ values of 9.8 mg $L^{-1}$ and 6.6 mg $L^{-1}$ of cadmium after 24 h and 48 h of exposure for embryos of *Pagrus major* (red sea bream), while for $LC_{50}$ values for larvae were 18.9 mg $L^{-1}$, 16.2 mg $L^{-1}$, 8 mg $L^{-1}$ and 5.6 mg $L^{-1}$ after 24, 48, 72 and 96 h of exposure at a temperature of 18 ºC and a salinity of 33. The $LC_{50}$ values reported above for hatching success are higher when compared to the values recorded in the present study (**Table 2.1**). Nonetheless, it must be highlighted that the above-mentioned studies referred to total cadmium, rather than to free ionic cadmium. It must be highlighted that although the present study monitored the endpoints for newly hatched sole larvae, these originated from embryos, which were also exposed to cadmium. As such, sole larvae were potentially more susceptible to the toxicant due to their internal burden. Therefore, data comparison with other studies monitoring the effect of cadmium exposure in marine larvae (e.g., Cao et al. (2009)) should be performed with caution and not overlook this issue.

Malformations on sole larvae after exposure to cadmium may indicate that this compound can bear a teratogenic risk (**Figure 2.3**). Cheng et al. (2000) reports teratogenic effects such as cardiac edema, hypopigmentation, tail malformations and yolk sac deformities, from cadmium exposure on embryos and larvae of the freshwater fish, *Danio rerio* after 28 h of fertilization, contributing to the idea that cadmium can disrupt developmental pathways. In the present study, the lower percentage of larvae displaying malformations after 72 and 96 h of exposure was due to the precocious death of specimens bearing malformations, rather than a lower toxicity of cadmium.

### 2.5.5. Single Cell Gel Electrophoresis Assay (Comet assay)

Genotoxicity is one the most relevant endpoints in risk assessment (Hayashi et al., 2005; Sarkar et al., 2015). Herein, this endpoint was assessed in all three species after exposure to cadmium. An increase in DNA damage was observed with increasing $Cd^{2+}$ concentrations, even when no significant effects were observed at the individual level in acute and chronic toxicity tests (**Figure 2.4**). As previously mentioned, cadmium genotoxicity has been assessed for several aquatic organisms in previous studies (Cambier et al., 2010; Chandurvelan et al., 2013b; Chang et al., 2009; Desai et al., 2006; Migliarini et al., 2005; Sarkar et al., 2015). However, cadmium has been proven to be a weak genotoxicant (Bertin and Averbeck, 2006). Cadmium genotoxicity is mainly appraised as an indirect effect caused by oxidative stress due to the production of reactive oxygen species (ROS) that cause DNA and protein oxidation, as well as DNA single strand breaks that may eventually lead to pathology or cell apoptosis (Bertin and

Averbeck, 2006; Nzengue et al., 2011; Shi et al., 2005). For instance, Lee et al. (2008) reported the overexpression of the detoxifying enzyme glutathione *S*-transferase (GST) in the marine copepod *Tigriopus japonicus* exposed to cadmium. This enzyme prevents lipid peroxidation due to ROS presence, acting as a catalyst between reduced glutathione (GSH) and cytotoxic compounds. Chang et al. (2009) reported an increase in DNA damage and olive tail moment, in heamocytes and hepatopancreas cells of *Litopenaeus vannamei* (white shrimp) due to the action of ROS. Therefore, it is possible that the exposure to $Cd^{2+}$ impairs the upregulation of GST allowing the accumulation of ROS and consequent cell damage. Cadmium-induced deformities in sole were similar to those previously observed in embryos and zebrafish larvae (*Danio rerio*) (Cheng et al., 2000). The authors concluded that even at lower concentrations (112 µg $L^{-1}$), cadmium affects specific gene regulators and causes a disruption of developmental events e.g. apoptosis and differentiation. Literature shows that $Cd^{2+}$ affects the structure and function of important proteins and induces DNA damage, as well as inhibits DNA repair processes (Bertin and Averbeck, 2006; Rossman et al., 1992).

It must be highlighted that total cadmium concentrations tested in the acute toxicity bioassays of all three organisms, *A. tonsa*, *P. varians* and *S. senegalensis*, were significantly higher when compared to those already determined or predicted to occur in the environment; nonetheless the range of concentrations selected to estimate cadmium's toxicity to the ELS of *A. tonsa*, the Fish Embryo Toxicity, as well as for the Comet assay for all organisms, is well within the range of values recorded for contaminated estuaries and coastal areas (Andres et al., 2000; Roast et al., 2001; World Health Organization, 2003). The current study has shown that environmentally relevant cadmium concentrations can promote a decrease in copepods' egg viability and an increase in fish larvae malformations, which may ultimately lead to effects at a population level. Even at concentrations where no effects were observed at an individual level, cadmium promoted genotoxicity to the organism at subcellular level, thus revealing the potential that the comet assay holds to be used as an early warning indicator.

## 2.6. Conclusions

The current study demonstrated the existence of significant effects of cadmium exposure on different species of marine organisms, negatively affecting survival and larval development of early life stages and adult specimens. Moreover, DNA damage at a

cellular level was recorded, even at concentrations where apparently no deleterious effects were observed using different endpoints. Therefore, by solely employing lethal and sublethal endpoints to monitor the toxicity of cadmium (or any other toxicant) researchers may overlook environmental pollution and end up with a biased perception of ecological risk. In other words, the negative effect of toxicants may be underestimated. By combining these tests with genotoxicity studies it is possible to develop early indicators of environmental pollution and therefore improve protocols to perform reliable risk assessment studies. Sensitivity between species shows that the copepod *A. tonsa* appears to be more sensitive to cadmium when compared to the shrimp *P. varians* and the sole *S. senegalensis*, thus likely being a better sentinel species for environmental pollution. As *A. tonsa* occupies a lower trophic level in marine systems than *P. varians* and *S. senegalensis*, deleterious effects promoted by cadmium contamination may easily trigger bottom-up cascading effects in marine trophic interactions. Overall, our study demonstrates the relevance of addressing cadmium toxicity in seawater due to its speciation. In order to encourage the development of synthesis studies (please see Hampton and Parker (2011)), we emphasize the urgent need to establish clear guidelines for experimental design and set up, as well as data reporting, for toxicity tests targeting metals due to their chemical speciation in seawater

## 2.7. References

Ajiboye, O.O., Yakubu, A.F., Adams, T.E., Olaji, E.D., Nwogu, N.A., 2010. A review of the use of copepods in marine fish larviculture. Rev Fish Biol Fisheries 21, 225–246. doi:10.1007/s11160-010-9169-3

Amirthalingam, T., Velusamy, G., Pandian, T.J., 2013. Cadmium-induced changes in mitotic index and genotoxicity on *Vigna unguiculata* (Linn.) Walp. J Environ Chem Ecotoxicol 5, 57–62. doi:10.5897/JECE11.008

Andres, S., Ribeyre, F., Tourencq, J.N., Boudou, A., 2000. Interspecific comparison of cadmium and zinc contamination in the organs of four fish species along a polymetallic pollution gradient (Lot River, France). Sci Total Environ 248, 11–25. doi:10.1016/S0048-9697(99)00477-5

Araujo, M.J., Reis, P., Braga, A.C., Antunes, C., 2013. Metal levels and accumulation patterns of sea lamprey ammocoetes (*Petromyzon marinus* Linnaeus, 1758) from a tidal freshwater habitat. Annales De Limnologie-International Journal of Limnology 49, 107–112. doi:10.1051/limn/2013044

Bambang, Y., Charmantier, G., Thuet, P., Trilles, J.P., 1995. Effect of cadmium on survival and osmoregulation of various developmental stages of the shrimp *Penaeus japonicus* (Crustacea: Decapoda). Mar Biol 123, 443–450. doi:10.1007/BF00349223

Bejarano-Escobar, R., Blasco, M., DeGrip, W.J., Oyola Velasco, J.A., Martín Partido, G., Francisco Morcillo, J., 2010. Eye development and retinal differentiation in an altricial fish species, the senegalese sole (*Solea senegalensis*, Kaup 1858). Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 314B, 580–605. doi:10.1002/jez.b.21363

Bertin, G., Averbeck, D., 2006. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). Biochimie 88, 1549–1559. doi:10.1016/j.biochi.2006.10.001

Breitholtz, M., Wollenberger, L., Dinan, L., 2003. Effects of four synthetic musks on the life cycle of the harpacticoid copepod *Nitocra spinipes*. Aquat Toxicol 63, 103–118. doi:10.1016/S0166-445X(02)00159-5

Burger, J., 2008. Assessment and management of risk to wildlife from cadmium. Sci Total Environ 389, 37–45. doi:10.1016/j.scitotenv.2007.08.037

Burke, J., Handy, R.D., Roast, S.D., 2003. Effect of low salinity on cadmium accumulation and calcium homeostasis in the shore crab (*Carcinus maenas*) at fixed free $Cd^{2+}$ concentrations. Environ Toxicol Chem 22, 2761–2767. doi:10.1897/02-410

Calado, R., Pimentel, T., Vitorino, A., Dionísio, G., Dinis, M.T., 2008. Technical improvements of a rearing system for the culture of decapod crustacean larvae, with emphasis on marine ornamental species. Aquaculture 285, 264–269. doi:10.1016/j.aquaculture.2008.08.019

Calado, R., Vitorino, A., Dionísio, G., Dinis, M.T., 2007. A recirculated maturation system for marine ornamental decapods. Aquaculture 263, 68–74. doi:10.1016/j.aquaculture.2006.10.013

Cambier, S., Gonzalez, P., Durrieu, G., Bourdineaud, J.-P., 2010. Cadmium-induced genotoxicity in zebrafish at environmentally relevant doses. Ecotoxicol Environ Safety 73, 312–319. doi:10.1016/j.ecoenv.2009.10.012

Cao, L., Huang, W., Shan, X., Xiao, Z., Wang, Q., Dou, S., 2009. Cadmium toxicity to embryonic-larval development and survival in red sea bream *Pagrus major*. Ecotoxicol Environ Safety 72, 1966–1974. doi:10.1016/j.ecoenv.2009.06.002

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2013a. Biochemical biomarker responses of green-lipped mussel, *Perna canaliculus*, to acute and subchronic waterborne cadmium toxicity. Aquat Toxicol 140, 303–313. doi:10.1016/j.aquatox.2013.06.015

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2013b. Waterborne cadmium impacts immunocytotoxic and cytogenotoxic endpoints in green-lipped mussel, *Perna canaliculus*. Aquat Toxicol 142, 283–293. doi:10.1016/j.aquatox.2013.09.002

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2012. Impairment of green-lipped mussel (*Perna canaliculus*) physiology by waterborne cadmium: relationship to tissue bioaccumulation and effect of exposure duration. Aquat Toxicol 124-125, 114–124. doi:10.1016/j.aquatox.2012.07.013

Chang, M., Wang, W.-N., Wang, A.-L., Tian, T.-T., Wang, P., Zheng, Y., Liu, Y., 2009. Effects of cadmium on respiratory burst, intracellular $Ca^{2+}$ and DNA damage in the white shrimp *Litopenaeus vannamei*. Comp Biochem Physiol C 149, 581–586. doi:10.1016/j.cbpc.2008.12.011

Cheng, S.H., Wai, A., So, C.H., Wu, R., 2000. Cellular and molecular basis of cadmium-induced deformities in zebrafish embryos. Environ Toxicol Chem 19, 3024–3031. doi:10.1897/1551-5028(2000)019<3024:CAMBOC>2.0.CO;2

Chiodi-Boudet, L., Polizzi, P., Romero, M.B., Robles, A., Gerpe, M., 2013. Lethal and sublethal effects of cadmium in the white shrimp *Palaemonetes argentinus*: a comparison between populations from contaminated and reference sites. Ecotoxicol Environ Safety 89, 52–58. doi:10.1016/j.ecoenv.2012.11.008

Cripe, G.M., 1994. Comparative Acute Toxicities of Several Pesticides and Metals to *Mysidopsis bahia* and Postlarval *Penaeus duorarum*. Environ Toxicol Chem 13, 1867–1872. doi:10.1002/etc.5620131119

De Lisle, P.F., Roberts, M.H., 1994. The effect of salinity on cadmium toxicity in the estuarine mysid *Mysidopsis bahia*: roles of osmoregulation and calcium. Mar Environ Res 37, 47–62. doi:10.1016/0141-1136(94)90062-0

De Lisle, P.F., Roberts, M.H., Jr., 1988. The effect of salinity on cadmium toxicity to the estuarine mysid *Mysidopsis bahia*: role of chemical speciation. Aquat Toxicol 12, 357–370. doi:10.1016/0166-445X(88)90062-8

Desai, S.R., Verlecar, X.N., Nagarajappa, Goswami, U., 2006. Genotoxicity of cadmium in marine diatom *Chaetoceros tenuissimus* using the alkaline Comet assay. Ecotoxicology 15, 359–363. doi:10.1007/s10646-006-0076-2

Dinis, M.T., 1992. Aspects of the potential of *Solea senegalensis* Kaup for aquaculture: larval rearing and weaning to an artificial diet. Aquacult Res 23, 515–520. doi:10.1111/j.1365-2109.1992.tb00795.x

Dinis, M.T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. Aquaculture 176, 27–38. doi:10.1016/S0044-8486(99)00047-2

Duthie, S.J., Collins, A.R., 1997. The influence of cell growth, detoxifying enzymes and DNA repair on hydrogen peroxide-mediated DNA damage (measured using the comet assay) in human cells. Free Radic. Biol. Med. 22, 717–724.

Eaton, J.G., McKim, J.M., Holcombe, G.W., 1978. Metal toxicity to embryos and larvae of seven freshwater fish species--I. Cadmium. Bull Environ Contam Toxicol 19, 95–103.

Elinder, C.G., 1985. Cadmium: uses, occurrence and intake. Cadmium and Health: a toxicological and epidimiological appraisal, Florida.

Engel, D.W., Fowler, B.A., 1979. Factors influencing cadmium accumulation and its toxicity to marine organisms. Environ Health Perspect 28, 81–88.

Environment Programme, U.N., 2008. Draft final review of scientific information on cadmium.

EPA, U.S., 1996. Ecological Effects Test Guidelines Mysid Acute Toxicty Test, Environmental Protection Agency.

Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The Comet assay for the evaluation of genotoxic impact in aquatic environments. Mutation Research/Reviews in Mutation Research 681, 80–92. doi:10.1016/j.mrrev.2008.03.001

Gustafsson, J.P., 2013. Visual MINTEQ ver. 3.0/3.1 [WWW Document]. Visual MINTEQ -

A free equilibrium speciation model. URL http://vminteq.lwr.kth.se/.

Hampton, S.E., Parker, J.N., 2011. Collaboration and Productivity in Scientific Synthesis. Bioscience 61, 900–910. doi:10.1525/bio.2011.61.11.9

Hayashi, M., Kamata, E., Hirose, A., Takahashi, M., Morita, T., Ema, M., 2005. In silico assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 588, 129–135. doi:10.1016/j.mrgentox.2005.09.009

Hayes, A.W., Kruger, C.L., 2014. Hayes' Principles and Methods of Toxicology, Sixth Edition. CRC Press.

Howard, C.L., Hacker, C.S., 1990. Effects of Salinity, Temperature, and Cadmium on Cadmium-Binding Protein in the Grass Shrimp, *Palaemonetes pugio*. Arch Environ Contam Toxicol 19, 341–347. doi:10.1007/BF01054976

Hutchinson, T.H., Williams, T.D., Eales, G.J., 1994. Toxicity of cadmium, hexavalent chromium and copper to marine fish larvae (*Cyprinodon variegatus*) and copepods (*Tisbe battagliai*). Mar Environ Res 38, 275–290. doi:10.1016/0141-1136(94)90028-0

International Agency for Research on Cancer, 1993. IARC. Cadmium and cadmium compounds. International Agency for Research on Cancer.

ISO, 2013. Water quality - Calanoid copepod early - life stage test with *Acartia tonsa*, International Organisation for Standardization.

ISO, 1999. Water quality - Determination of acute lethal toxicity to marine copepods (Copepoda, Crustacea), International Organisation for Standardization.

Leung, K.M., Morritt, D., Wheeler, J.R., Whitehouse, P., Sorokin, N., Toy, R., Holt, M., Crane, M., 2001. Can saltwater toxicity be predicted from freshwater data? Marine Poll Bull 42, 1007–1013.

Mance, G., 1987. Pollution threat of heavy metals in aquatic environments.

Mhadhbi, L., Boumaiza, M., Beiras, R., 2010. A standard ecotoxicological bioassay using early life stages of the marine fish *Psetta maxima*. Aquatic Living Resources 23, 209–216. doi:10.1051/alr/2010014

Migliarini, B., Campisi, A.M., Maradonna, F., Truzzi, C., Annibaldi, A., Scarponi, G., Carnevali, O., 2005. Effects of cadmium exposure on testis apoptosis in the marine teleost *Gobius niger*. Gen. Comp. Endocrinol. 142, 241–247. doi:10.1016/j.ygcen.2004.12.012

Mohammed, A., 2013. Why are Early Life Stages of Aquatic Organisms more Sensitive to Toxicants than Adults?, in: New Insights Into Toxicity and Drug Testing. InTech. doi:10.5772/55187

Moraïtou-Apostolopoulou, M., Verriopoulos, G., Lentzou, P., 1979. Effects of sublethal concentrations of cadmium as possible indicators of cadmium pollution for two populations of *Acartia clausi* (Copepoda) living at two differently polluted areas. Bull Environ Contam Toxicol 23, 642–649. doi:10.1007/BF01770019

Nigro, M., Frenzilli, G., Scarcelli, V., Gorbi, S., Regoli, F., 2002. Induction of DNA strand breakage and apoptosis in the eel *Anguilla anguilla*. Mar Environ Res 54, 517–520. doi:10.1016/S0141-1136(02)00178-2

Nogueira, P.R., Lourenco, J., Mendo, S., Rotchell, J.M., 2006. Mutation analysis of ras gene in the liver of European eel (*Anguilla anguilla* L.) exposed to benzo[a]pyrene. Marine Poll Bull 52, 1611–1616. doi:10.1016/j.marpolbul.2006.05.017

Nordberg, G.F., 2009. Historical perspectives on cadmium toxicology. Toxicol. Appl. Pharmacol. 238, 192–200. doi:10.1016/j.taap.2009.03.015

Nzengue, Y., Candéias, S.M., Sauvaigo, S., Douki, T., Favier, A., Rachidi, W., Guiraud, P., 2011. The toxicity redox mechanisms of cadmium alone or together with copper and zinc homeostasis alteration: its redox biomarkers. J Trace Elem Med Biol 25, 171–180. doi:10.1016/j.jtemb.2011.06.002

OECD, 2013. OECD Guidelines for the Testing of Chemicals, Section 2 Test No. 236: Fish Embryo Acute Toxicity (FET) Test. OECD Publishing.

Plhalová, L., Mácová, S., Doleželová, P., Maršálek, P., Svobodová, Z., Pištěková, V., Bedáňová, I., Voslářová, E., Modrá, H., 2011. Comparison of Terbutryn Acute Toxicity to *Danio rerio* and *Poecilia reticulata*. AVB 79, 593–598. doi:10.2754/avb201079040593

Rainbow, P.S., Malik, I., O'Brien, P., 1993. Physicochemical and physiological effects on the uptake of dissolved zinc and cadmium by the amphipod crustacean *Orchestia gammarellus*. Aquat Toxicol 25, 15–30. doi:10.1016/0166-445X(93)90017-U

Ray, S., 1984. Bioaccumulation of cadmium in marine organisms. Experientia 40, 14–23.

Roast, S.D., Widdows, J., Jones, M.B., 2001. Effects of salinity and chemical speciation on cadmium accumulation and toxicity to two mysid species. Environ Toxicol Chem 20, 1078–1084. doi:10.1002/etc.5620200519

Rodrigues, C.M., Bio, A., Amat, F., Vieira, N., 2011. Artisanal salt production in Aveiro/Portugal - an ecofriendly process. Saline Syst. 7, 3. doi:10.1186/1746-1448-7-3

Rombough, P.J., Garside, E.T., 1982. Cadmium Toxicity and Accumulation in Eggs and Alevins of Atlantic Salmon *Salmo salar*. Canadian Journal of Zoology 60, 2006–2014. doi:10.1139/z82-258

Rossman, T.G., Roy, N.K., Lin, W.C., 1992. Is cadmium genotoxic? IARC Sci. Publ. 367–375.

Sarkar, A., Bhagat, J., Ingole, B.S., Rao, D.P., Markad, V.L., 2015. Genotoxicity of cadmium chloride in the marine gastropod *Nerita chamaeleon* using comet assay and alkaline unwinding assay. Environ. Toxicol. 30, 177–187. doi:10.1002/tox.21883

Shi, H., Sui, Y., Wang, X., Luo, Y., Ji, L., 2005. Hydroxyl radical production and oxidative damage induced by cadmium and naphthalene in liver of *Carassius auratus*. Comp Biochem Physiol C 140, 115–121. doi:10.1016/j.cca.2005.01.009

Shields, R.J., Bell, J.G., Luizi, F.S., Gara, B., Bromage, N.R., Sargent, J.R., 1999. Natural Copepods Are Superior to Enriched *Artemia* Nauplii as Feed for Halibut Larvae (*Hippoglossus hippoglossus*) in Terms of Survival, Pigmentation and Retinal Morphology: Relation to Dietary Essential Fatty Acids. J. Nutr. 129, 1186–1194.

Silva, A.R.R., Cardoso, D.N., Cruz, A., Lourenco, J., Mendo, S., Soares, A.M.V.M., Loureiro, S., 2015. Ecotoxicity and genotoxicity of a binary combination of triclosan and carbendazim to *Daphnia magna*. Ecotoxicol Environ Safety 115, 279–290. doi:10.1016/j.ecoenv.2015.02.022

Støttrup, J.G., 2003. Production and nutritional value of copepods. Live Feeds in Marine Aquaculture. Blackwell Science.

Støttrup, J.G., 2000. The elusive copepods: their production and suitability in marine aquaculture. Aquacult Res 31, 703–711. doi:10.1046/j.1365-2109.2000.318488.x

Støttrup, J.G., Richardson, K., Kirkegaard, E., Pihl, N.J., 1986. The cultivation of *Acartia tonsa* Dana for use as a live food source for marine fish larvae. Aquaculture 52, 87–96. doi:10.1016/0044-8486(86)90028-1

Støttrup, J.G., Shields, R., Gillespie, M., Gara, M.B., Sargent, J.R., Bell, J.G., Henderson, R.J., Tocher, D.R., Sutherland, R., Noess, T., Jensen, A.M., Naas, K., Meeren, T.V.D., Harboe, T., Sanchez, F.J., Sorgeloos, P., Dhert, P., Fitzgerald, R., Hendry, C., 1998. The production and use of copepods in larval rearing of halibut, turbot and cod. Bulletin of the Aquaculture Association of Canada 41–45.

Sunda, W.G., Engel, D.W., Thuotte, R.M., 1978. Effect of Chemical Speciation on Toxicity of Cadmium to Grass Shrimp, *Palaemonetes pugio*: Importance of Free Cadmium Ion. Environ. Sci. Technol. 12, 409–413. doi:10.1021/es60140a003

Sykes, A.V., Domingues, P.M., Andrade, J.P., 2006. Effects of Using Live Grass Shrimp (*Palaemonetes varians*) as the only Source of Food for the Culture of Cuttlefish, *Sepia officinalis* (Linnaeus, 1758). Aquaculture International 14, 551–568. doi:10.1007/s10499-006-9054-1

Thompson, J., Bannigan, J., 2008. Cadmium: Toxic effects on the reproductive system and the embryo. Reprod Toxicol 25, 304–315. doi:10.1016/j.reprotox.2008.02.001

Turner, D.R., Whitfield, M., Dickson, A.G., 1981. The equilibrium speciation of dissolved components in freshwater and sea water at 25°C and 1 atm pressure. Geochimica et Cosmochimica Acta 45, 855–881. doi:10.1016/0016-7037(81)90115-0

Verriopoulos, G., Moraïtou-Apostolopoulou, M., 1982. Differentiation of the sensitivity to copper and cadmium in different life stages of a copepod. Marine Poll Bull 13, 123–125. doi:10.1016/0025-326X(82)90368-X

World Health Organization, 2003. Cadmium Review (No. 1).

Zirino, A., Yamamoto, S., 1972. A pH-dependent model for the chemical speciation of copper, zinc, cadmium and lead in seawater. Limnol Oceanogr, 17, 5, 661-671.

## 2.8. Supplementary Data

**Table S2.1:** Chemical speciation of cadmium under salinity 20 and temperature 20 ºC using the equilibrium model vMinteq ver. 3.0/3.1 for the copepod *Acartia tonsa*. *n.d. – not determined, concentration of this species contributed less than 0.01% of total cadmium concentration.

| ***Acartia tonsa*** | | | | | | |
|---|---|---|---|---|---|---|
| Acute Toxicity Test | | | | | | |
| Nominal Concentration (mg $L^{-1}$) | Salinity | Temperature (ºC) | Total Estimated Cadmium Concentration (mg $L^{-1}$) | | | |
| | | | $Cd^{2+}$ | $CdOH^{+}$ | $CdCl^{+}$ | $CdCl_2$ (aq) |
| 0.16 | 20 | 20 | 0.01 | 0.00002 | 0.09 | 0.05 |
| 0.28 | | | 0.02 | 0.00003 | 0.17 | 0.09 |
| 0.50 | | | 0.04 | 0.00006 | 0.30 | 0.16 |
| 0.90 | | | 0.08 | 0.00011 | 0.54 | 0.28 |
| 1.62 | | | 0.14 | 0.00019 | 0.97 | 0.50 |
| ELS Test | | | | | | |
| Nominal Concentration (µg $L^{-1}$) | Salinity | Temperature (ºC) | Total Estimated Cadmium Concentration (µg $L^{-1}$) | | | |
| | | | $Cd^{2+}$ | $CdOH^{+}$ | $CdCl^{+}$ | $CdCl_2$ (aq) |
| 6.87 | 20 | 20 | 0.59 | 0.0008 | 4.13 | 2.14 |
| 13.75 | | | 1.19 | 0.0017 | 8.27 | 4.28 |
| 27.5 | | | 2.39 | 0.0033 | 16.53 | 8.57 |
| 55 | | | 4.79 | 0.0066 | 33.07 | 17.14 |
| 110 | | | 9.57 | 0.0132 | 66.13 | 34.28 |
| SCGE Assay (Comet Assay) | | | | | | |
| Nominal Concentration (µg $L^{-1}$) | Salinity | Temperature (ºC) | Total Estimated Cadmium Concentration (µg $L^{-1}$) | | | |
| | | | $Cd^{2+}$ | $CdOH^{+}$ | $CdCl^{+}$ | $CdCl_2$ (aq) |
| 6.87 | 20 | 20 | 0.59 | 0.0008 | 4.13 | 2.14 |
| 27.5 | | | 2.39 | 0.0033 | 16.53 | 8.57 |
| 110 | | | 9.57 | 0.0132 | 66.13 | 34.28 |

**Table S2.2:** Chemical speciation of cadmium under salinity 35 and temperature 20 ºC using the equilibrium model vMinteq ver. 3.0/3.1 for the ditch shrimp *Palaemon varians*. *n.d. – not determined, concentration of this species contributed less than 0.01% of total cadmium concentration.

| ***Palaemon varians*** | | | | | | |
|---|---|---|---|---|---|---|
| Acute Toxicity Test | | | | | | |
| Nominal Concentration (mg $L^{-1}$) | Salinity | Temperature (ºC) | Total Estimated Cadmium Concentration (mg $L^{-1}$) | | | |
| | | | $Cd^{2+}$ | $CdOH^{+}$ | $CdCl^{+}$ | $CdCl_2$ (aq) |
| 0.3 | 35 | 20 | 0.01 | n.d. | 0.16 | 0.13 |
| 0.6 | | | 0.03 | n.d. | 0.31 | 0.26 |
| 1.2 | | | 0.05 | n.d. | 0.63 | 0.52 |
| 2.4 | | | 0.11 | n.d. | 1.25 | 1.04 |
| 4.8 | | | 0.22 | n.d. | 2.51 | 2.07 |
| 9.6 | | | 0.43 | n.d. | 5.02 | 4.14 |
| SCGE Assay (Comet Assay) | | | | | | |
| Nominal Concentration (mg $L^{-1}$) | Salinity | Temperature (ºC) | Total Estimated Cadmium Concentration (mg $L^{-1}$) | | | |
| | | | $Cd^{2+}$ | $CdOH^{+}$ | $CdCl^{+}$ | $CdCl_2$ (aq) |
| 0.3 | 35 | 20 | 0.01 | n.d. | 0.16 | 0.13 |
| 0.6 | | | 0.03 | n.d. | 0.31 | 0.26 |
| 1.2 | | | 0.05 | n.d. | 0.63 | 0.52 |

**Table S2.3** Chemical speciation of cadmium under salinity 35 and temperature 18 ºC using the equilibrium model vMinteq ver. 3.0/3.1 for the sole *Solea senegalensis*. *n.d. – not determined, concentration of this species contributed less than 0.01% of total cadmium concentration.

| ***Solea senegalensis*** | | | | | | |
|---|---|---|---|---|---|---|
| Fish Embryo Toxicity Test (Acute Toxicity) | | | | | | |
| Nominal Concentration ($mg\ L^{-1}$) | Salinity | Temperature (ºC) | Total Estimated Cadmium Concentration ($mg\ L^{-1}$) | | | |
| | | | $Cd^{2+}$ | $CdOH^{+}$ | $CdCl^{+}$ | $CdCl_2$ (aq) |
| 0.5 | 35 | 18 | 0.02 | n.d. | 0.26 | 0.21 |
| 1 | | | 0.04 | n.d. | 0.52 | 0.43 |
| 2 | | | 0.09 | n.d. | 1.05 | 0.86 |
| 4 | | | 0.18 | n.d. | 2.10 | 1.72 |
| 8 | | | 0.36 | n.d. | 4.19 | 3.44 |
| 12 | | | 0.55 | n.d. | 6.29 | 5.16 |
| Fish Embryo Toxicity Test | | | | | | |
| Nominal Concentration ($\mu g\ L^{-1}$) | Salinity | Temperature (ºC) | Total Estimated Cadmium Concentration ($\mu g\ L^{-1}$) | | | |
| | | | $Cd^{2+}$ | $CdOH^{+}$ | $CdCl^{+}$ | $CdCl_2$ (aq) |
| 6.25 | 35 | 18 | 0.28 | n.d. | 3.28 | 2.69 |
| 12.5 | | | 0.57 | n.d. | 6.55 | 5.38 |
| 25 | | | 1.14 | n.d. | 13.11 | 10.75 |
| 50 | | | 2.27 | n.d. | 26.22 | 21.50 |
| 75 | | | 3.41 | n.d. | 39.33 | 32.25 |
| 100 | | | 4.54 | n.d. | 52.44 | 43.01 |
| SCGE Assay (Comet Assay) | | | | | | |
| Nominal Concentration ($\mu g\ L^{-1}$) | Salinity | Temperature (ºC) | Total Estimated Cadmium Concentration ($\mu g\ L^{-1}$) | | | |
| | | | $Cd^{2+}$ | $CdOH^{+}$ | $CdCl^{+}$ | $CdCl_2$ (aq) |
| 1.56 | 35 | 18 | 0.07 | n.d. | 0.82 | 0.67 |
| 3.12 | | | 0.14 | n.d. | 1.64 | 1.34 |
| 6.25 | | | 0.28 | n.d. | 3.28 | 2.69 |
| 12.5 | | | 0.57 | n.d. | 6.55 | 5.38 |

# Chapter 3

# Influence of environmental conditions on the toxicokinetics of cadmium in the marine copepod *Acartia tonsa*

Submitted

# Influence of environmental conditions on the toxicokinetics of cadmium in the marine copepod *Acartia tonsa*

## 3.1. Abstract

Marine and estuarine ecosystems are highly productive areas that often act as a final sink for several pollutants, such as cadmium. Environmental conditions in these habitats can affect metal speciation, as well as its uptake and depuration by living organisms. The aim of this study was to assess cadmium uptake and depuration rates in the euryhaline calanoid copepod *Acartia tonsa* under different pH, salinity and temperature conditions. Cadmium speciation did not vary with a changing pH or temperature, but cadmium free ion concentration and activity decreased with increasing salinity. Cadmium concentration in *A. tonsa* increased with increasing pH during the uptake phase registering a peak at the intermediate pH of 7.5, while the depuration rate fluctuated with increasing pH. Cadmium uptake increased with temperature, while the depuration rate also increased from 15 to 20 ºC, but displayed an opposite trend decreasing when temperature increased from 20 to 25 ºC. Cadmium uptake was not affected by salinity, although the depuration rate increased with a higher salinity. The present study shows that cadmium uptake and depuration rates in the marine copepod *A. tonsa* were more affected by biological processes, mainly driven by metabolic mechanisms, rather than by metal speciation in the exposure medium.

Keywords: uptake rate, depuration rate, metal, abiotic factors, bioconcentration, chemical speciation

## 3.2. Introduction

Zooplankton plays a key role in the trophic webs of marine and brackish ecosystems, being paramount in the bottom-up reallocation of trace elements (Fisher et al., 2000). Copepods are well represented in the zooplankton of marine ecosystems, being widely distributed and often dominating coastal blooms (Xu et al., 2001). These organisms have been used in standardized ecotoxicological studies for several years, as they are considered to be sensitive indicators of metal pollution (Bao et al., 2013; Barka et al., 2010; Moraïtou-Apostolopoulou et al., 1979; Pedroso et al., 2007; Toudal and Riisgard, 1987; Xu et al., 2001).

Metals are common environmental pollutants and are considered to be hazardous for marine organisms due to their persistence in water or sediment, as well as due to their high bioaccumulation potential (Mohammed et al., 2011). Previous studies have demonstrated that the accumulation of metals in marine invertebrates can differ significantly between species and under varying environmental conditions (Aksu, 2001; Mubiana and Blust, 2007; Philp, 2001; Xu et al., 2012). Different water characteristics, such as the concentration of suspended organic matter, calcium and magnesium concentration, zinc concentration, redox potential, salinity, temperature or pH, may affect the toxicity of metals (Amirthalingam et al., 2013; Di Toro et al., 2001; Engel and Fowler, 1979; Environment Programme, 2008; Frazier, 1979; Panda and Panda, 2002; Ray, 1984). Effects of a toxicant on the organism are related to the way uptake takes place, to what extent it is being accumulated, distributed to different body compartments, stored or metabolized and subsequently eliminated.

Cadmium is considered one of the most toxic metals to aquatic organisms (Howard and Hacker, 1990). In order to fully understand the process of cadmium bioavailability, it is important to evaluate its uptake and depuration kinetics in organisms. The uptake of metals such as cadmium, cobalt or copper is known to be influenced by the availability of their free ionic form, which in turn is determined by salinity, temperature and pH (Burke et al., 2003; Mubiana and Blust, 2007; Roast et al., 2001). Mubiana and Blust (2007) demonstrated that as temperature increased the uptake and depuration rates of two non-essential metals, cadmium and lead in the marine bivalve *Mytilus edulis* also increased. Cadmium uptake in the Asiatic clam, *Corbicula fluminea*, was significantly decreased with decreasing pH (from 7.8 to 5.0), while a positive correlation was found between cadmium uptake and increasing temperature (Graney, 1984). Mercury (Hg(II)) accumulation in the

shore crab *Carcinus maenas* was found to be favored at lower salinities (Laporte et al., 1997).

Studies addressing toxicokinetics (TK) are commonly employed to describe and explain the way metal exposure can be associated to effects in the organism over time. TK studies provide information on the way the metal is being absorbed from the surrounding media and excreted from the body, as well as how toxicity develops over time (Directorate-General for Health and Food Safety and European Commission, 2013). The outcome of TK studies can provide a better understanding on how the organism is processing the chemical, enabling the calculation of uptake and depuration rate constants along with the half-life time of the chemical in the organism (i.e. residence time) (Directorate-General for Health and Food Safety and European Commission, 2013). By simulating short-term exposure conditions, TK studies can be considered a useful tool that may allow data extrapolation among species and exposure times in order to assist risk assessment (Ashauer and Escher, 2010) and regulatory procedures for protecting environmental and human health (Dorne and Renwick, 2005).

The present study aimed to determine the bioconcentration potential of cadmium in a marine calanoid copepod under different environmental conditions. In this way, the uptake and depuration kinetics of cadmium were experimentally evaluated for *Acartia tonsa* stocked under different pH, salinity and temperature conditions by employing a first-order one-compartment TK model.

## 3.3. Materials and Methods

### 3.3.1. Copepod Culture

Cultures of the marine calanoid copepod *Acartia tonsa* were kept under a continuous life cycle using artificial seawater (ASW) prepared by mixing freshwater purified by a reverse osmosis unit with the commercial marine salts Tropic Marin® Pro Reef (Tropic Marin, Wartenberg, Germany) according to the instructions provided by the manufacturer. Cultures were started from eggs kindly provided by Escola Superior de Tecnologia do Mar, IPL, Peniche, Portugal. Copepod eggs were stocked in 15-L poly(methyl methacrylate) (PMMA) cylindroconical tanks supplied with constant aeration (~3 bubbles $s^{-1}$) at a salinity of 20±1, a temperature of 20±1 ºC and a photoperiod of 16 h light: 08 h dark. After hatching, different developmental stages (nauplii, copepodites and adults) of *A. tonsa* were separated in different 15-L PMMA tanks using appropriate mesh screens to

retain each developmental life stage. Organisms were fed daily ad libitum with the cryptophyte *Rhodomonas lens* CCMP 739 (at a minimum stock density of ~2 x $10^7$ cells $mL^{-1}$). The density of adult copepods under culture was kept at a maximum of ~130 specimens $L^{-1}$, with culture tanks being siphoned daily to collect eggs and remove excess of food and debris (e.g., dead organisms and fecal pellets). Water was fully renewed once a week, with collected eggs being stored at 4 ºC and used to start new *A. tonsa* cultures whenever necessary (Drillet et al., 2006).

### 3.3.2. Test Chemical

Cadmium chloride anhydrous (CAS No. 10108-64-2, Sigma-Aldrich, Germany) was selected to perform the trials to determine the bioconcentration potential of cadmium in *A. tonsa*. A stock solution of 100 mg Cd $L^{-1}$ was prepared with ultrapure water using a Millipore® Academic Milli-Q system. Test concentrations were achieved through dilution in artificial seawater (ASW). Chemical analysis screening for cadmium in the ultrapure water was performed using Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). Samples from the stock solution and from the concentration in ultrapure water were acidified after spiking and sent to LCA (Central Laboratory of Analysis, University of Aveiro, Portugal) to assess and confirm contamination accuracy. The chemical equilibrium model Visual MINTEQ ver. 3.0/3.1 (Gustafsson, 2013) was used to calculate the speciation of cadmium in ASW medium using the concentration of all salt constituents (information supplied by the manufacturer of the commercial marine salts employed in the present study). From the total cadmium concentration, the parameters derived and used for data analysis were the cadmium free ion concentration and the cadmium free ion activity for each environmental condition tested. Three exposure conditions were tested for each parameter: 7.0, 7.5 and 7.9 for pH, 10, 20 and 30 for salinity and 15, 20 and 25 ºC for temperature. The target pH levels were achieved through direct $CO_2$ diffusion in the seawater and maintained with the use of a pressure regulator that would control the amount of $CO_2$ dosed in the aquariums according to the value set on the controller. Climatic chambers wer used in order to control target temperature in the medium, while salinity levels were obtained by dilutions and salinity was measured daily to assure it did not deviate from levels initially set. For pH conditions, temperature was fixed at 20 ºC, while salinity was fixed at 20. For salinity conditions, temperature was fixed at 20 ºC, while pH varied according to salinity. For temperature conditions, salinity was fixed to 20 and pH

to 7.9. The Visual MINTEQ ver. 3.0/3.1 chemical equilibrium model calculated the ionic strength of each test solution.

### 3.3.3. Bioconcentration Tests

For the bioconcentration tests, a concentration of 6.88 µg of Cd $L^{-1}$ was used, corresponding to the Lowest Observed Effect Concentration (LOEC) of cadmium on the hatching success of *A. tonsa*; this value was obtained from an Early Life Stage test previously performed (Pavlaki et al., 2016). Bioconcentration experiments consisted of two phases; an uptake phase where the organisms were exposed to cadmium through water contamination and a depuration/elimination phase where the organisms were allowed to depurate when stocked in a non-contaminated medium. Copepods were acclimated for 24 hours to each environmental condition prior to testing. The uptake and depuration phases lasted 48 hours each for all of exposure conditions tested. Copepods were not fed during the experiment in order to minimize possible interference from algae (uptake/adsorption). During the uptake phase 400-500 adult *A. tonsa* were exposed to cadmium in three replicate 4-L glass aquariums and then transferred to clean medium for the depuration phase. Sampling times varied for each condition tested, with 6-8 samplings being performed during the uptake phase and 4-5 during the depuration phase. Every sampling consisted of three replicates, with ~30 copepods being pooled per replicate.

### 3.3.4. Chemical Analysis

Each replicate sample of *A. tonsa* was rinsed with ultrapure water to remove excess medium, freeze-dried for 24 hours, weighed on a microbalance and then digested. Digestions were performed with a mixture of $HNO_3$ and $HClO_4$, at a ratio of 7:1 (v/v, Baker Ultrex II Ultra Pure) using four heating steps (step 1: 85 ºC for 60 minutes, step 2: 130 ºC for 60 minutes, step 3: 160 ºC for 60 minutes and step 4: 180 ºC until dryness) in order to destroy all organic material. Residues were taken up in 200 µL of 0.1M $HNO_3$ (Baker Ultrex II Ultra Pure). Cadmium concentration was measured using Graphite Furnace Atomic Absorption Spectrophotometry (Perkin-Elmer 5100 PC). For every digestion cycle, 3-6 replicates of blanks and 3 replicates of certified reference material (CRM) (DOLT-5, Dogfish liver CRM for trace metals and other constituents) were used to control for the accuracy of the method (cadmium concentration, 14.5 mg $kg^{-1}$, SD=0.6 mg $kg^{-1}$).

Detection limit was 0.052 µg Cd $L^{-1}$ (n=20). Recovery of cadmium from the certified reference material was 119% (SD=2.3%).

### 3.3.5. Toxicokinetics Model

The uptake and depuration kinetics of cadmium in the copepods was described using a first-order one-compartment model considering the organism as one singular compartment.

Background cadmium body concentration in the control organisms was significantly lower than the detection limit; therefore $C_0$ was fixed to 0 and was not included in these equations.

For the uptake phase the model used reads:

$Q(t) = \frac{k_1}{k_2} * C_e * (1 - e^{(-k_2 * t)})$ (**Eq. 3.1**)

And for the depuration phase:

$Q(t) = \frac{k_1}{k_2} * C_e * (e^{(-k_2 * (t - t_c))} - e^{(-k_2 * t)})$ (**Eq. 3.2**)

where Q(t) is the concentration in the organism in µg Cd $g^{-1}$ dry body weight at time t,

$k_1$ is the uptake rate constant in $mL_{medium}\ g_{organism}^{-1}\ hour^{-1}$,

$k_2$ is the depuration rate constant in $hour^{-1}$,

$C_e$ is the exposure concentration in the medium in µg Cd $L^{-1}$,

$t_c$ is the time when the organisms where transferred to fresh uncontaminated medium in hours, and

t is the sampling time in hours.

Both equations used for describing the cadmium uptake and depuration patterns were fitted simultaneously as suggested by the OECD guideline 305 on fish bioaccumulation testing (2012).

### 3.3.6. Statistical Analysis

Kinetics parameters for each environmental condition were estimated using non-linear regression analysis by fitting uptake and depuration equations to the data using SPSS Statistics Package (version 20). Significance of differences in $k_1$ and $k_2$ between exposure conditions was tested applying a Generalized Likelihood Ratio Test.

The time (expressed in hours) that organisms required to eliminate half the amount of cadmium ($DT_{50}$), and the bioconcentration factor (BCF) in $mL_{medium}$ $g_{organism}^{-1}$ were calculated as:

$DT_{50} = \frac{\ln(2)}{k_2}$ (**Eq. 3.3**),

$BCF = \frac{k_1}{k_2}$ (**Eq. 3.4**)

## 3.4. Results

### 3.4.1. Chemical Analysis

Cadmium concentrations measured in the stock and test solutions did not differ more than 2-15% from the nominal ones, thus confirming the accuracy of the spiking technique. The cadmium free ion concentrations and cadmium free ion activities for each environmental condition estimated with the Visual MINTEQ equilibrium model, are presented in **Table 3.1**. Cadmium free ion concentrations as well as cadmium free ion activities were fairly similar across the ranges of pH and temperatures tested but decreased with increasing salinity of the test solution.

**Table 3.1** Percentage of the total cadmium concentration present as free cadmium ions, cadmium free ion concentration and cadmium free ion activity at different pH, salinity and temperature levels of exposure used to determine the influence of environmental conditions on cadmium uptake kinetics in *Acartia tonsa*. Test solutions were spiked with a nominal cadmium concentration of 6.88 µg $L^{-1}$, and measured concentrations did not differ more than 15% from the nominal ones. Cadmium free ion concentrations and activities were calculated with the equilibrium model Visual Minteq ver. 3.0/3.1 (Gustafsson, 2013), using the nominal cadmium concentration, the ionic composition of the test medium and the test conditions as the starting point.

| Environmental Parameters | | | Percentage of Ionic Cadmium (%) | Cadmium Free Ion Concentration (µg $L^{-1}$) | Cadmium Free Ion Activity (µg $L^{-1}$) |
|---|---|---|---|---|---|
| **Temperature (ºC)** | **pH** | **Salinity** | | | |
| | **7.0** | | 8.7 | 0.60 | 0.17 |
| **20** | **7.5** | **20** | 8.7 | 0.60 | 0.17 |
| | **7.9** | | 8.7 | 0.60 | 0.17 |
| | **7.8** | **10** | 15.9 | 1.10 | 0.36 |
| **20** | **7.9** | **20** | 8.7 | 0.60 | 0.17 |
| | **7.9** | **30** | 5.5 | 0.38 | 0.11 |
| **15** | | | 8.8 | 0.61 | 0.18 |
| **20** | **7.9** | **20** | 8.7 | 0.60 | 0.17 |
| **25** | | | 8.6 | 0.60 | 0.17 |

### 3.4.2. Bioconcentration Tests

No copepod mortality was observed during the 48-hour cadmium uptake phase in any of the bioassays performed. Mortality ranged from 5-12% at the end of all seven bioassays, with the bioassay performed at 25 ºC displaying the highest mortality after 96 hours (data not shown). Uptake and depuration kinetics calculated using cadmium free ion activities (**Table 3.1)** are presented in **Table 3.2**. For further comparison, kinetics parameters derived using total cadmium and cadmium free ion concentrations can be found in Supplementary Data (**Table S3.1**). Depuration rate constants ($k_2$) were independent of the type of data used in the model: total cadmium concentration, cadmium free ion concentration or cadmium free ion activity; therefore $k_2$ values and 95% confidence intervals presented are similar for the different ways of expressing exposure concentrations in all conditions described below.

**pH**

Cadmium concentrations in the copepods body did not reach steady state within 48 hours of uptake at any of the three different pH values used in the bioconcentration tests. The internal concentration of cadmium increased at different rates during the uptake phase. Mean cadmium concentration in the copepods was 12.8 µg Cd $g^{-1}$ at pH 7.0, 19.4 µg Cd $g^{-1}$ at pH 7.5 and 14.6 µg Cd $g^{-1}$ at pH 7.9 after 48 hours of uptake (**Figure 3.1**). As pH increased the uptake rate increased as well, however, it did not follow a clear pattern. Depuration rate was faster at the highest pH (**Figure 3.1**).

The kinetics parameters for cadmium free ion activity obtained by a first-order one-compartment model are presented in **Table 3.2**. Uptake rate constant $k_1$ was significantly lower at pH 7 compared to higher pH levels ($X^2_{(1)}$=18.49-25.18, $p<0.001$), but did not differ between the two highest pH levels (7.5 and 7.9) ($X^2_{(1)}$=0.40, n.s. for ionic activity). Depuration rate constant $k_2$ was significantly higher at the highest pH compared to lower pH levels ($X^2_{(1)}$=16.40-18.92, $p<0.001$), while at the two lowest pH levels no difference was observed ($X^2_{(1)}$=0.83, n.s.). As no steady state was reached in any of the treatments, bioconcentration factors were calculated from the ratio of $k_1$ and $k_2$ values. The BCF value was 526x$10^3$ $mL_{medium}$ $g_{organism}^{-1}$ for pH 7, 10627x$10^3$ $mL_{medium}$ $g_{organism}^{-1}$ for pH 7.5 and 132x$10^3$ $mL_{medium}$ $g_{organism}^{-1}$ for pH 7.9. The time needed for the organisms to eliminate half the amount of cadmium ($DT_{50}$) was estimated at 231 hours for pH 7, 2310 hours for pH 7.5 and 31 hours for pH 7.9.

**Table 3.2** Uptake and elimination kinetic parameters for the bioaccumulation of cadmium in the copepod *Acartia tonsa* exposed to Cd-spiked artificial seawater, no food provided. Kinetics parameters were calculated using a one-compartment model (**Equations 3.1** and **3.2**), with estimated cadmium ion free activity in the test solutions (**Table 3.1**) as the exposure concentration; 95% confidence intervals are shown in brackets. Different letters indicate significant differences between kinetics parameters estimated for a certain environmental condition (likelihood ratio test; $p<0.05$).

| Environmental Parameters | $k_1$ ($mL_{water}\ g_{organism}^{-1}$, $hour^{-1}$) | $k_2$ ($hour^{-1}$) | BCF ($x10^3$) | $DT_{50}$ (hours) |
|---|---|---|---|---|
| **pH** | | | | |
| **7.0** | 1579[a] (1190-1968) | 0.003[a] (0.000-0.010) | 526 | 231 |
| **7.5** | 3188[b] (2833-3544) | 0.0003[a] (0.000-0.003) | 10627 | 2310 |
| **7.9** | 2904[b] (2279-3529) | 0.022[b] (0.013-0.030) | 132 | 31 |
| **Salinity** | | | | |
| **10** | 2127[a] (1895-2359) | 0.007[ac] (0.004-0.010) | 304 | 99 |
| **20** | 2904[a] (2196-3613) | 0.022[b] (0.012-0.031) | 132 | 31 |
| **30** | 2637[a] (1640-3633) | 0.018[bc] (0.004-0.031) | 146 | 38 |
| **Temperature (ºC)** | | | | |
| **15** | 1323[a] (951-1694) | 0.003[a] (0.00-0.010) | 441 | 231 |
| **20** | 2904[b] (2262-3547) | 0.022[b] (0.013-0.030) | 132 | 31 |
| **25** | 4893[c] (4354-5431) | 0.012[b] (0.009-0.016) | 408 | 57.8 |

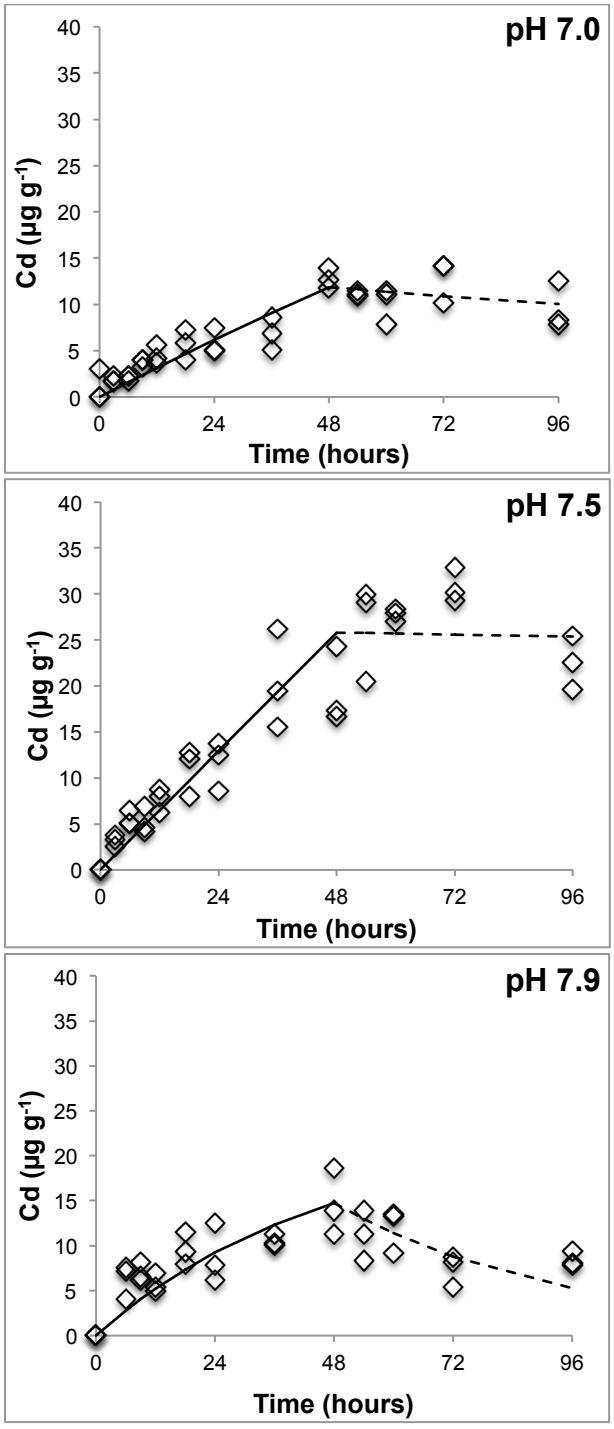


**Figure 3.1** Uptake and elimination kinetics of cadmium in the marine copepod *Acartia tonsa* at different pH levels. Diamonds represent real data, continuous lines the fit of the uptake model (**Equation 1**) to the data, dotted lines the fit of the depuration model (**Equation 2**). Modeling used the estimated cadmium free ion activity in the test solutions reported in Table 1 as the measure of exposure during the uptake phase.

**Salinity**

No steady state was reached for cadmium concentration in the copepods exposed at different salinities. Mean cadmium concentrations in *A. tonsa* decreased with increasing salinity and were 25.0, 14.6 and 8.6 µg Cd $g^{-1}$ at salinities of 10, 20 and 30, respectively (**Figure 3.2**). The uptake rate constant $k_1$ increased with increasing salinity when total cadmium concentration was used for modeling (**Table S3.1**), but did not differ among treatments when based on cadmium free ion concentrations and cadmium free ion activities in the test solutions (**Table 3.2 and Table S3.1**; ($X^2_{(1)}$=0.19-0.96, n.s. for cadmium free ion concentration, $X^2_{(1)}$=0.40-3.13, n.s. for cadmium free ion activity). Depuration rate constant $k_2$ was significantly higher at the intermediate salinity compared to the lower one ($X^2_{(1)}$=6.08, $p<0.05$), but did not differ between the other salinities ($X^2_{(1)}$=0.44-2.37, n.s., respectively). Bioconcentration factors calculated as $k_1/k_2$ for cadmium free ion activity were $304x10^3$, $10627x10^3$ and $132x10^3$ $mL_{medium}$ $g_{organism}^{-1}$ at salinities of 10, 20 and 30, respectively. At these salinities, $DT_{50}$ for cadmium depuration was 99, 31 and 38 hours, respectively.

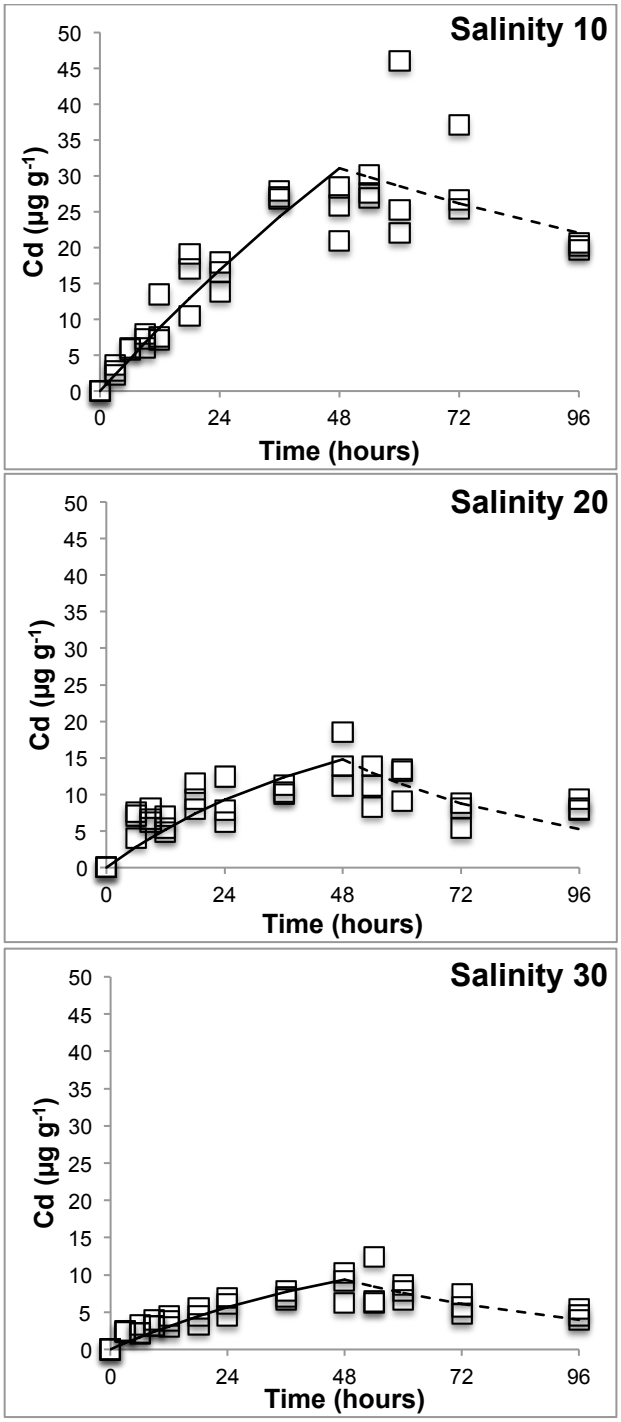


**Figure 3.2** Uptake and elimination kinetics of cadmium in the marine copepod *Acartia tonsa* at different salinities. Squares represent real data, continuous lines the fit of the uptake model (**Equation 1**) to the data, dotted lines the fit of the depuration model (**Equation 2**). Modeling used the estimated cadmium free ion activity in the test solutions reported in Table 1 as the measure of exposure during the uptake phase.

**Temperature**

In none of the three temperature exposures cadmium concentrations in the organisms reached steady state. After 48 hours of exposure, mean cadmium concentration in the copepods was 11.6, 14.6, and 31.1 µg Cd $g^{-1}$ at temperatures of 15, 20 and 25 ºC, respectively (**Figure 3.3**). There was a 3-fold and 2-fold increase in the cadmium body concentration when temperature increased from 15 to 25 ºC and from 20 to 25 ºC, respectively. As temperature increased, the uptake rate also increased. Cadmium depuration rate increased from 15 ºC to 20 ºC, while at 25 ºC it was slightly lower (**Figure 3.3**). The kinetics parameters obtained with the first-order one-compartment model are presented in **Table 3.2**. Uptake rate $k_1$ differed significantly between temperatures ($X^2_{(1)}$= 11.51-55.57, $p<0.001$ for cadmium free ion activity). Depuration rate $k_2$ did not differ between 20 ºC and 25 ºC ($X^2_{(1)}$=3.02, n.s.), but was significantly lower at 15 ºC compared to 20 ºC and 25 ºC ($X^2_{(1)}$=14.26, $p<0.001$ and $X^2_{(1)}$=4.65, $p<0.05$, respectively). BCF values were 441x$10^3$, 132x$10^3$ and 408x$10^3$ $mL_{medium}$ $g_{organism}^{-1}$ for the cadmium free ion activity at temperatures of 15, 20 and 25 ºC, respectively. $DT_{50}$ values at these temperatures were estimated to be 231, 31 and 57.8 hours, respectively.

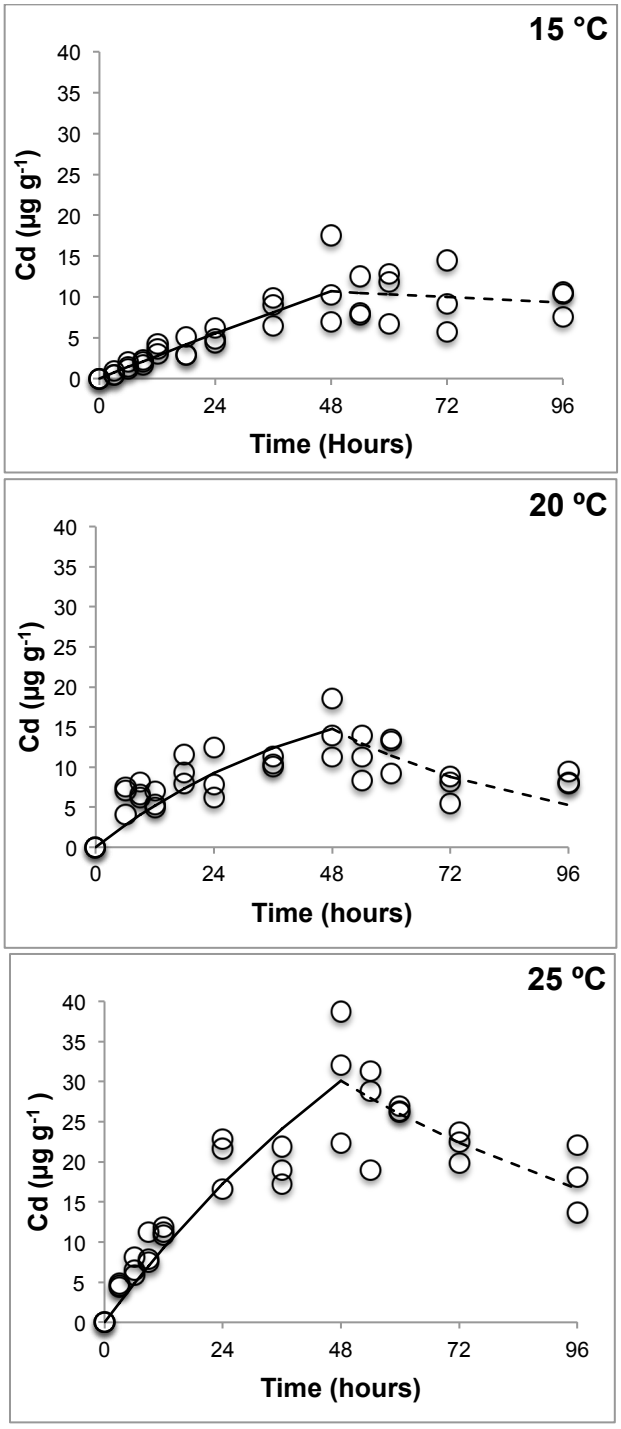


**Figure 3.3** Uptake and elimination kinetics of cadmium in the marine copepod *Acartia tonsa* at different temperatures. Circles represent real data, continuous lines the fit of the uptake model (**Equation 1**) to the data, dotted lines the fit of the depuration model (**Equation 2**). Modeling used the estimated cadmium free ion activity in the test solutions reported in Table 1 as the measure of exposure during the uptake phase.

## 3.5. Discussion

The present study determined the effect of different environmental conditions, namely pH, salinity and temperature, on the toxicokinetics of cadmium in the marine copepod *Acartia tonsa*. The copepods were not fed during the experiments and starvation did not seem to significantly influence their physiology, as confirmed by the low mortality in our tests. According to Finiguerra et al (2013), copepods can tolerate starvation for up to ~15 days until mortality peaks to 100%. The same authors have also shown that during the first days of starvation copepods rely on previous nutritional provisioning (e.g. catabolism of energy reserves) to reproduce and survive and are therefore physiologically unaffected by food deprivation. Cadmium accumulation in the marine copepod was significantly affected by each environmental parameter when compared to the culture conditions of the organism. According to Rainbow (1998) and Ray (1984), crustaceans cannot regulate their body concentration of non-essential metals, such as cadmium, which leads to a higher body concentration, high storage capacity and slow depuration of cadmium, if excreted at all. Cadmium accumulation and detoxification in the organism depends mainly on the presence of low molecular weight proteins with high affinity binding sites, known as metalloproteins or metallothioneins, which are normally induced as a response to exposure and are capable of forming stable complexes with cadmium (Rainbow, 2007). In that way, metals bound to metallothioneins are stored and prevented from possibly causing damage, e.g. in the DNA (Ray, 1984). Preston (1973), after analyzing a large set of data, observed that BCFs for cadmium uptake in plankton and specifically crustaceans could reach levels as high as $10^4$ and $10^3$, respectively, which are in agreement with the values found for *A. tonsa* in the present study. Furthermore, we also showed that the highest BCF values were attained as pH, salinity and temperature decreased. This can be attributed to the slow depuration of cadmium from the copepods due to the metal being stored rather than excreted. Low depuration rates may indicate the presence of a possible storage compartment, such as the oil sac (Lee et al., 2006; van den Bosch and Gabriel, 1994), which could potentially cause metal concentrations to accumulate above the organisms threshold and eventually lead to mortality. This scenario may lead to high $DT_{50}$ values, ranging from 31 hours up to 96 days. Sick and Baptist (1979) reported an average turnover rate for cadmium in the copepod *Pseudodiaptomus pelagicus* (formerly *P. coronatus*) of 40 hours at a temperature of 20 ºC, pH 7.2 and salinity 25. Denton et al. (1981) found that cadmium half-life in the oyster *Saccostrea echinata* strongly depends on temperature and salinity fluctuations and reported values ranging from 30 to 85 days.

Taking into consideration that the average lifespan of the marine copepod *A. tonsa* is approximately 80 days, higher $DT_{50}$ values seem unrealistic, as the organism would die before being able to eliminate the excess of cadmium. In this way, it is legitimate to say that the potential of *A. tonsa* to transfer cadmium to higher trophic levels is high. Nonetheless, to the authors' knowledge and despite the trophic relevance of the copepod *A. tonsa* in marine food webs, data on bioaccumulation of cadmium is still missing for this species.

**pH**

The internal cadmium concentration in copepods showed a non-linear relationship with pH. This is in line with the findings reported by Goetze et al. (2014), who found that at the lowest and highest pH (7.7 and 8.2, respectively), cadmium was accumulated in oysters in a similar way, while at pH 7.9 its accumulation decreased by half. Cadmium uptake rate showed a decrease as pH decreased. This trend appears to be contradictory to the hypothesis that cadmium bioavailability increases with a lowering pH due to the dissociation of cadmium from creating complexes and consequently increasing $Cd^{2+}$ free ion concentration. However, the decrease in pH resulted in a higher $H^+$ activity and increased positively charged groups, e.g. amino groups, and decreased the negatively charged groups by protonation, e.g. carboxyl and phosphate (Wang et al., 2016). This setting could eventually lead to a higher competition of protons with $Cd^{2+}$ ions at the membrane binding sites. This is in agreement with the concept of the Biotic Ligand Model (Di Toro et al., 2001; Paquin et al., 2002). A similar explanation was used by Martins et al. (2004) to explain the finding that cadmium uptake in aquatic moss increased with increasing pH from 3 to 5. Likewise, Xu et al. (Xu et al., 2012) found that when two marine phytoplanktonic species were exposed to cadmium under decreasing pH levels the bioavailable cadmium was reduced, thus concluding that the uptake of cadmium was more likely being affected by the protonation of the binding cellular surface. The marine copepods, *A. tonsa*, showed low depuration rates at the three pH levels tested in the present study. Xu et al. (2001) reported that cadmium was lost mainly through feces, rather than being eliminated through excretion in the calanoid copepod *Calanus sinicus*. The same authors also mention that the egestion rate of cadmium was mainly affected by food concentration. Thus, an increased food ingestion rate would likely result in an increase in cadmium egestion rate. However, since copepods were not fed during the experiments, this could have contributed to the low depuration rates recorded at the three pH levels that were tested in the present study. When comparing different pH levels, a

higher depuration rate at the highest pH level implies faster cadmium depuration, which could be explained by the animal's physiology. Normal culture conditions for *A. tonsa* were at a pH of 7.9±0.1, so that a decrease in pH could be considered as a stressor to its physiological state. Overall, this condition may have forced the copepods to maintain homeostasis by increasing its energetic demand (Cripps et al., 2014) at the expense of other metabolic processes, such as metal elimination. Consequently, both uptake and depuration of cadmium are mostly being mediated by biological processes, with metal speciation likely playing a minor role at tested pH levels. This assumption is in line with the small variation in cadmium free ion activity recorded at pH levels tested in the present study (**Table 3.1**).

**Salinity**

Cadmium uptake rate constant $k_1$ was not affected by salinity, but the internal cadmium concentration increased as salinity decreased due to the increased availability of cadmium ($Cd^{2+}$) (**Table 3.1**). Cadmium is known to have a strong affinity to chloride ions ($Cl^-$) to create complexes, rendering it less bioavailable, and/or affecting its competition with sodium ions ($Na^+$) at the membrane's binding sites (Di Toro et al., 2001; Newman, 2014; Paquin et al., 2002). Previous studies have shown that when salinity decreases from 35 to 25, and even to 15, respiration of *A. tonsa*, decreased, possibly as a way to cope with changes in osmoregulation (Gaudy et al., 2000; Kinne, 1964). A similar effect was described by Hutcheson (1974), for blue crabs (*Callinectes sapidus*). The author reported that cadmium concentration was higher at lower salinities due to the increase in metabolic energy required to maintain an osmotic gradient and therefore allowed a lower allocation of energy to control the metal influx to the crab's tissues. Again, biological processes such as the organism's metabolic rates/respiration play a key role in cadmium uptake, since the similarity in the uptake rates could not be explained from the higher concentration of free $Cd^{2+}$ ions at the lower salinity of the exposure medium. Another biological process that may have allowed $Cd^{2+}$ to enter the cells is the $Ca^{2+}$-transport system through $Ca^{2+}$ channels due to its similar ionic radius and charge (Bjerregaard and Depledge, 1994). At low salinities, $Cd^{2+}$ competes with $Ca^{2+}$ due to the response of marine crustaceans to ionoregulate, as mentioned above, by increasing or decreasing ionic uptake when salinity diverges from normal. Therefore, a decrease/increase in salinity may result in an up- or downregulation of $Ca^{2+}$ diffusion from the medium and the opportunistic transfer of $Cd^{2+}$ through the same channels. Several studies have shown that cadmium accumulation in marine organisms at different salinities, such as in crabs (Burke et al., 2003), sponges

(Philp, 2001), mussels (Bjerregaard and Depledge, 1994) and fish (Cinier et al., 1999) partly depends on $Ca^{2+}$ transport channels. This suggests that the competition between the two divalent cations, $Cd^{2+}$ and $Ca^{2+}$, is likely taking place at the biotic ligand sites of the organism. No difference was observed in cadmium depuration rates of *A. tonsa* exposed to cadmium at the two highest salinities, while at a salinity of 10 the depuration rate was lower. Turnover rates of cadmium in the copepod *Calanus sinicus* were in agreement with the values in this study, with copepods being exposed to a salinity of 30 and a temperature of 20 ºC (Xu and Wang, 2001). According to Gaudy (2000), *A. tonsa* showed no significant differences in metal excretion between salinities of 30 and 20, while when salinity dropped to 15 the excretion rate decreased significantly . This trend could explain the low depuration rate of cadmium from the copepods in our study. The abovementioned low metal excretion at lower salinities had already been identified in the copepod *A. tonsa*, as well as in estuarine crabs *Carcinus maena* and *Uca rapax*, and is considered to be a mechanism that euryhaline organisms use to regulate and maintain isosmotic body fluids when osmotic changes occur (Gaudy et al., 2000; Wright, 1977; Zanders and Rojas, 1996).

**Temperature**

As temperature increased, the uptake rate of cadmium as well as the internal cadmium concentration in the copepod *A. tonsa* increased. Cadmium assimilation in copepods showed a 3-fold increase when temperature shifted from 15 to 25 ºC. These findings are consistent with several studies that confirm an increased cadmium uptake and accumulation rate with increasing temperatures in a number of marine taxa (e.g., marine bivalves (Ali and Taylor, 2010; Mubiana and Blust, 2007), marine crustaceans (O'Hara, 1973; White and Rainbow, 1986)). At higher temperatures, the metabolic rate of the organism increases and with that protein synthesis, which can result in enhanced consumption, assimilation and increased formation of metal-binding proteins (e.g. metallothioneins) in the presence of metals. Howard and Hacker (Howard and Hacker, 1990) reported that an increase in temperature also resulted in an increase in the level of metallothionein-like cadmium binding proteins in the estuarine ditch shrimp (*Palaemonetes pugio*) when exposed to cadmium. These authors highlight the role played by metal-binding proteins, which are considered a defense/detoxification mechanism against metal toxicity. In the present study the depuration rate increased with temperature from 15 to 20 ºC, while no significant difference was recorded on the depuration rate when temperature was raised from 20 to 25 ºC. This trend can be attributed to the faster

metabolism of *A. tonsa* at higher temperatures. A study on the respiratory and excretion rates of *A. tonsa* under different temperatures and salinities showed that both parameters decreased with decreasing temperature (Gaudy et al., 2000) as a result of decreasing the metabolic energetic demand by the organism.

## 3.6. Conclusions, implications and future perspectives

The present study found that biological processes, possibly metabolism/respiration, are paramount in cadmium uptake and depuration rates in the marine copepod *Acartia tonsa* exposed to different environmental conditions. In general, *A. tonsa* appears to have a high capacity to bioaccumulate cadmium under the environmental conditions tested. The low depuration rates recorded may indicate the existence of a potential storage compartment, which could subsequently cause metal concentrations to reach above the organisms' threshold and eventually lead to mortality. In this way, the metal uptake and storage capacity displayed by marine copepods can pose a threat to higher trophic levels in food webs through the occurrence of food-chain transfer.

The experimental design employed in the present study was able to assess the influence of three environmental factors individually on the patterns of cadmium accumulation in this copepod. It can be used as a foundation for a more in-depth understanding on how cadmium accumulation may vary under fluctuating environmental factors. Future studies should consider going further and assess more complex and realistic scenarios, such as the joint or interacting effects of different abiotic factors on the uptake and depuration of cadmium. Such approach could then be used for the development of predictive models that may improve the accuracy of risk assessment within the frame of multiple environmental scenarios.

## 3.7. References

Aksu, Z., 2001. Equilibrium and kinetic modelling of cadmium(II) biosorption by *C. vulgaris* in a batch system: effect of temperature. Separation and Purification Technology 21, 285–294.

Ali, M., Taylor, A., 2010. The effect of salinity and temperature on the uptake of cadmium and zinc by the common blue mussel, *Mytilus edulis* with some notes on their survival. Mesopot. J. Mar. Sci. 25, 11–30.

Amirthalingam, T., Velusamy, G., Pandian, T.J., 2013. Cadmium-induced changes in mitotic index and genotoxicity on *Vigna unguiculata* (Linn.) Walp. Journal of Environmental Chemistry and Ecotoxicology 5, 57–62. doi:10.5897/JECE11.008

Ashauer, R., Escher, B.I., 2010. Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. J Environ Monit 12, 2056–2061. doi:10.1039/c0em00234h

Bao, V.W.W., Leung, K.M.Y., Lui, G.C.S., Lam, M.H.W., 2013. Acute and chronic toxicities of Irgarol alone and in combination with copper to the marine copepod *Tigriopus japonicus*. Chemosphere 90, 1140–1148. doi:10.1016/j.chemosphere.2012.09.022

Barka, S., Pavillon, J.-F., Amiard-Triquet, C., 2010. Metal distributions in *Tigriopus brevicornis* (Crustacea, Copepoda) exposed to copper, zinc, nickel, cadmium, silver, and mercury, and implication for subsequent transfer in the food web. Environ. Toxicol. 25, 350–360. doi:10.1002/tox.20505

Bjerregaard, P., Depledge, M.H., 1994. Cadmium accumulation in *Littorina littorea*, *Mytilus edulis* and *Carcinus maenas*: the influence of salinity and calcium ion concentrations. Mar. Biol. 119, 385–395. doi:10.1007/BF00347535

Burke, J., Handy, R.D., Roast, S.D., 2003. Effect of low salinity on cadmium accumulation and calcium homeostasis in the shore crab (*Carcinus maenas*) at fixed free $Cd^{2+}$ concentrations. Environ Toxicol Chem 22, 2761–2767. doi:10.1897/02-410

Cinier, C.D., Petit-Ramel, M., Faure, R., Garin, D., Bouvet, Y., 1999. Kinetics of cadmium accumulation and elimination in carp *Cyprinus carpio* tissues. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 122, 345–352. doi:10.1016/S0742-8413(98)10132-9

Cripps, G., Lindeque, P., Flynn, K.J., 2014. Have we been underestimating the effects of ocean acidification in zooplankton? Glob Chang Biol 20, 3377–3385. doi:10.1111/gcb.12582

Denton, G.R.W., Burdon-Jones, C., 1981. Influence of temperature and salinity on the uptake, distribution and depuration of mercury, cadmium and lead by the black-lip

oyster *Saccostrea echinata*. Mar. Biol. 64, 317–326. doi:10.1007/BF00393633

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ Toxicol Chem 20, 2383–2396. doi:10.1002/etc.5620201034/full

Directorate-General for Health and Food Safety, European Commission, E.C., 2013. Assessing the New Challenged for Risk Assessment. European Commission. doi:10.2772/37863

Dorne, J.L.C.M., Renwick, A.G., 2005. The refinement of uncertainty/safety factors in risk assessment by the incorporation of data on toxicokinetic variability in humans. Toxicol. Sci. 86, 20–26. doi:10.1093/toxsci/kfi160

Drillet, G., Iversen, M.H., Sørensen, T.F., Ramløv, H., Lund, T., Hansen, B.W., 2006. Effect of cold storage upon eggs of a calanoid copepod, *Acartia tonsa* (Dana) and their offspring. Aquaculture 254, 714–729. doi:10.1016/j.aquaculture.2005.11.018

Engel, D.W., Fowler, B.A., 1979. Factors influencing cadmium accumulation and its toxicity to marine organisms. Environ Health Perspect 28, 81–88.

Environment Programme, U.N., 2008. Draft final review of scientific information on cadmium.

Finiguerra, M.B., Dam, H.G., Avery, D.E., Burris, Z., 2013. Sex-specific tolerance to starvation in the copepod *Acartia tonsa*. J Exp Mar Biol Ecol 446, 17–21. doi:10.1016/j.jembe.2013.04.018

Fisher, N.S., Stupakoff, I., Sanudo-Wilhelmy, S., Wang, W.X., Teyssié, J.L., Fowler, S.W., Crusius, J., 2000. Trace metals in marine copepods: a field test of a bioaccumulation model coupled to laboratory uptake kinetics data. Mar. Ecol. Prog. Ser. 194, 211–218. doi:10.3354/meps194211

Frazier, J.M., 1979. Bioaccumulation of cadmium in marine organisms. Environ Health Perspect 28, 75–79.

Gaudy, R., Cervetto, G., Pagano, M., 2000. Comparison of the metabolism of *Acartia clausi* and *A. tonsa*: influence of temperature and salinity. J Exp Mar Biol Ecol 247, 51–65.

Goetze, S., Matoo, O.B., Beniash, E., Saborowski, R., Sokolova, I.M., 2014. Interactive effects of $CO_2$ and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. Aquat Toxicol 149, 65–82. doi:10.1016/j.aquatox.2014.01.027

Graney, R., 1984. The influence of substrate, pH, diet and temperature upon cadmium accumulation in the asiatic clam (*Corbicula fluminea*) in laboratory artificial streams.

Water Res. 18, 833–842. doi:10.1016/0043-1354(84)90267-7

Gustafsson, J.P., 2013. Visual MINTEQ ver. 3.0/3.1 [WWW Document]. Visual MINTEQ - A free equilibrium speciation model. URL http://vminteq.lwr.kth.se/ (accessed 9.9.15).

Howard, C.L., Hacker, C.S., 1990. Effects of Salinity, Temperature, and Cadmium on Cadmium-Binding Protein in the Grass Shrimp, *Palaemonetes pugio*. Arch Environ Contam Toxicol 19, 341–347. doi:10.1007/BF01054976

Hutcheson, M.S., 1974. The effect of temperature and salinity on cadmium uptake by the blue crab, *Callinectes sapidus*. Chesapeake Science 15, 237–241. doi:10.2307/1350976

Kinne, O., 1964. The effects of salinity and temperature on marine and brackish water animals—II. Salinity and salinity-temperature combinations. A. Rev. Oceanogr. mar. Biol.

Laporte, J.M., Truchot, J.P., Ribeyre, F., Boudou, A., 1997. Combined effects of water pH and salinity on the bioaccumulation of inorganic mercury and methylmercury in the shore crab *Carcinus maenas*. Marine Poll Bull 34, 880–893. doi:10.1016/S0025-326X(97)00059-3

Lee, R.F., Hagen, W., Kattner, G., 2006. Lipid storage in marine zooplankton. Mar. Ecol. Prog. Ser.

Martins, R.J.E., Pardo, R., Boaventura, R.A.R., 2004. Cadmium(II) and zinc(II) adsorption by the aquatic moss *Fontinalis antipyretica*: effect of temperature, pH and water hardness. Water Res. 38, 693–699. doi:10.1016/j.watres.2003.10.013

Mohammed, E.H., Wang, G., Xu, Z., Liu, Z., Wu, L., 2011. Physiological response of the intertidal copepod *Tigriopus japonicus* experimentally exposed to cadmium. AACL Bioflux.

Moraïtou-Apostolopoulou, M., Verriopoulos, G., Lentzou, P., 1979. Effects of sublethal concentrations of cadmium as possible indicators of cadmium pollution for two populations of *Acartia clausi* (Copepoda) living at two differently polluted areas. Bull Environ Contam Toxicol 23, 642–649. doi:10.1007/BF01770019

Mubiana, V.K., Blust, R., 2007. Effects of temperature on scope for growth and accumulation of Cd, Co, Cu and Pb by the marine bivalve *Mytilus edulis*. Mar Environ Res 63, 219–235. doi:10.1016/j.marenvres.2006.08.005

Newman, M.C., 2014. Fundamentals of Ecotoxicology. CRC Press.

OECD, 2012. OECD Guidelines for the Testing of Chemicals, Section 3: Degradation and Accumulation Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure. OECD Publishing. doi:10.1787/9789264185296-en

O'Hara, J., 1973. Cadmium Uptake by Fiddler Crabs Exposed to Temperature and Salinity Stress. J. Fish. Res. Bd. Can. 30, 846–848. doi:10.1139/f73-143

Panda, B.B., Panda, K.K., 2002. Genotoxicity and Mutagenicity of Metals in Plants, in: Physiology and Biochemistry of Metal Toxicity and Tolerance in Plants. Springer Netherlands, Dordrecht, pp. 395–414. doi:10.1007/978-94-017-2660-3_15

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G.C., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., McGeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B., 2002. The biotic ligand model: a historical overview. Comp Biochem Physiol C 133, 3–35.

Pavlaki, M.D., Araújo, M.J., Cardoso, D.N., Silva, A.R.R., Cruz, A., Mendo, S., Soares, A.M.V.M., Calado, R., Loureiro, S., 2016. Ecotoxicity and genotoxicity of cadmium in different marine trophic levels. Environ Pollut 215, 203–212. doi:10.1016/j.envpol.2016.05.010

Pedroso, M.S., Bersano, J.G.F., Bianchini, A., 2007. Acute silver toxicity in the euryhaline copepod *Acartia tonsa*: influence of salinity and food. Environ Toxicol Chem 26, 2158–2165. doi:10.1897/06-485R.1

Philp, R.B., 2001. Effects of experimental manipulation of pH and salinity on $Cd^{2+}$ uptake by the sponge *Microciona prolifera* and on sponge cell aggregation induced by $Ca^{2+}$ and $Cd^{2}$. Arch Environ Contam Toxicol 41, 282–288.

Preston, A., 1973. Cadmium in the marine environment of the United Kingdom. Marine Poll Bull 4, 105–107. doi:10.1016/0025-326X(73)90329-9

Rainbow, P.S., 2007. Trace metal bioaccumulation: models, metabolic availability and toxicity. Environ Int 33, 576–582. doi:10.1016/j.envint.2006.05.007

Rainbow, P.S., 1998. Phylogeny of trace metal accumulation in crustaceans, in: Langston, W.J., Bebianno, M.J. (Eds.), Metal Metabolism in Aquatic Environments. Springer US, Boston, MA, pp. 285–319. doi:10.1007/978-1-4757-2761-6_9

Ray, S., 1984. Bioaccumulation of cadmium in marine organisms. Experientia 40, 14–23.

Roast, S.D., Widdows, J., Jones, M.B., 2001. Effects of salinity and chemical speciation on cadmium accumulation and toxicity to two mysid species. Environ Toxicol Chem 20, 1078–1084. doi:10.1002/etc.5620200519

Sick, L.V., Baptist, G.J., 1979. Cadmium incorporation by the marine copepod *Pseudodiaptomus coronatus*. Limnology and Oceanography 24, 453–462. doi:10.4319/lo.1979.24.3.0453

Toudal, K., Riisgard, H.U., 1987. Acute and Sublethal Effects of Cadmium on Ingestion,

Egg-Production and Life-Cycle Development in the Copepod *Acartia tonsa*. Mar. Ecol. Prog. Ser. 37, 141–146. doi:10.3354/meps037141

van den Bosch, F., Gabriel, W., 1994. A model of growth and development in copepods. Limnology and Oceanography 39, 1528–1542. doi:10.4319/lo.1994.39.7.1528

Wang, Z., Meador, J.P., Leung, K.M.Y., 2016. Metal toxicity to freshwater organisms as a function of pH: A meta-analysis. Chemosphere 144, 1544–1552. doi:10.1016/j.chemosphere.2015.10.032

White, S.L., Rainbow, P.S., 1986. Accumulation of cadmium by *Palaemon elegans* (Crustacea: Decapoda). Mar. Ecol. Prog. Ser.

Wright, D.A., 1977. The Effect of Salinity on Cadmium Uptake by the Tissues of the Shore Crab *Carcinus Maenas*. J Exp Biol 67, 137–146.

Xu, Y., Shi, D., Aristilde, L., Morel, F.M.M., 2012. The effect of pH on the uptake of zinc and cadmium in marine phytoplankton: Possible role of weak complexes. Limnology and Oceanography 57, 293–304. doi:10.4319/lo.2012.57.1.0000

Xu, Y., Wang, W.-X., Hsieh, D.P.H., 2001. Influences of metal concentration in phytoplankton and seawater on metal assimilation and elimination in marine copepods. Environ Toxicol Chem 20, 1067–1077. doi:10.1002/etc.5620200518

Xu, Y., Wang, W.X., 2001. Individual responses of trace-element assimilation and physiological turnover by the marine copepod *Calanus sinicus* to changes in food quantity. Mar. Ecol. Prog. Ser. 218, 227–238.

Zanders, I.P., Rojas, W.E., 1996. Salinity effects on cadmium accumulation in various tissues of the tropical fiddler crab *Uca rapax*. Environ Pollut 94, 293–299. doi:10.1016/S0269-7491(96)00095-4

## 3.8. Supplementary Data

**Table S3.1** Uptake and elimination kinetic parameters for the bioaccumulation of cadmium in the copepod *Acartia tonsa* exposed to Cd-spiked artificial seawater (no food provided). Kinetics parameters were calculated using a one-compartment model (**Equations 3.1** and **3.2**), with total measured cadmium concentrations and cadmium free ion concentrations in the test solutions (**Table 3.1**) as the measure of exposure; $k_1t$, the uptake rate calculated with total cadmium concentration, $k_1f$, the uptake rate calculated with estimated cadmium free ion concentration; 95% confidence intervals are shown in brackets. Different letters indicate significant differences between kinetics parameters estimated for a certain environmental condition (likelihood ratio test; $p<0.05$).

| Environmental Parameters | $k_1t$ ($mL_{water}\ g_{organism}^{-1}$, $hour^{-1}$) | $k_1f$ ($mL_{water}\ g_{organism}^{-1}$, $hour^{-1}$) | $k_2$ ($hour^{-1}$) | BCFt ($x10^3$) | BCFf ($x10^3$) | $DT_{50}$ (hours) |
|---|---|---|---|---|---|---|
| **pH** | | | | | | |
| **7.0** | 39.0[a] (29.4-48.7) | 448[a] (338-559) | 0.003[a] (0.000-0.010) | 13 | 149 | 231 |
| **7.5** | 78.8[b] (70.0-87.6) | 905[b] (804-1006) | 0.0003[a] (0.000-0.003) | 263 | 3017 | 2310 |
| **7.9** | 71.8[b] (56.4-87.3) | 826[b] (648-1003) | 0.022[b] (0.013-0.030) | 3.3 | 37.5 | 31 |
| **Salinity** | | | | | | |
| **10** | 111[a] (99.2-124) | 697[a] (621-773) | 0.007[ac] (0.004-0.010) | 15.9 | 99.6 | 99 |
| **20** | 71.8[b] (54.3-89.3) | 826[a] (624-1024) | 0.022[b] (0.012-0.031) | 3.3 | 37.5 | 31 |
| **30** | 42.2[b] (26.2-58.1) | 765[a] (476-1055) | 0.018[bc] (0.004-0.031) | 2.4 | 42.5 | 38 |
| **Temperature (ºC)** | | | | | | |
| **15** | 34.6[a] (24.9-44.3) | 394[a] (283-504) | 0.003[a] (0.00-0.010) | 11.5 | 131 | 231 |
| **20** | 71.8[b] (55.9-87.7) | 826[b] (643-1008) | 0.022[b] (0.013-0.030) | 3.3 | 37.5 | 31 |
| **25** | 121[c] (108-134) | 1405[c] (1250-1560) | 0.012[b] (0.009-0.016) | 10.1 | 117 | 58 |

# Chapter 4

# Toxicokinetics of cadmium in *Palaemon varians* postlarvae under waterborne and/or dietary exposure

Submitted

# Toxicokinetics of cadmium in *Palaemon varians* postlarvae under waterborne and/or dietary exposure

## 4.1. Abstract

The present study assessed cadmium uptake and depuration rates in the euryhaline estuarine shrimp *Palaemon varians* under different exposure routes. Postlarval shrimp were exposed to cadmium concentrations through contaminated water, contaminated diet and a worst-case scenario where both contaminated water + diet were used. Results from this study showed that in the uptake phase, cadmium concentration in *P. varians* was the highest under the worst-case scenario. Cadmium uptake rate through water was faster when compared to the uptake rate from diet. Shrimp were unable to eliminate cadmium from their body, showing no depuration when exposed through different exposure routes. The present study highlights a possible existence of an inert fraction in the organism where cadmium is being stored and detoxified, but not eliminated by the shrimp.

Keywords: estuarine shrimp, bioconcentration, bioaccumulation, uptake rate, depuration rate, inert fraction

## 4.2. Introduction

Brackish water ecosystems, such as estuaries, are simultaneously affected by naturally changing biotic and abiotic conditions and by anthropogenic activities. Contaminants' emissions (e.g. metals, fertilizers, pesticides or polycyclic aromatic hydrocarbons) can have different sources, through river runoffs, as well as industrial, agricultural or domestic waste discharges (Goetze et al., 2014).

Recent studies on brackish water ecosystems have focused on the assessment of adverse effects induced by chemical contaminants, such as metals, in fish (Isani et al., 2009; Morcillo et al., 2016; Souid et al., 2013) and bivalves (Goetze et al., 2014; Rocha et al., 2015a; 2015b; Tan and Wang, 2012), as these organisms are often perceived as bioindicators for pollution (Viarengo and Canesi, 1991). Curiously, crustaceans receive significantly less attention by researchers (Chang et al., 2009) even though they are considered to be very sensitive to the impact of such contaminants when compared to other taxa (Bolton-Warberg et al., 2006; Howard and Hacker, 1990; Luo et al., 2015; Rajkumar et al., 2011).

When exposed to metal concentrations crustaceans are either able to regulate essential metals (e.g. zinc or copper) or store and subsequently detoxify non-essential metals (e.g. cadmium or silver) (Rainbow and White, 1989). The main organ in crustaceans for storage, detoxification and binding proteins, e.g. metallothioneins (MTs), is the hepatopancreas or midgut gland (Chang et al., 2009; Kaoud and Eldahshan, 2010) due to the existence of different types of cells responsible for those processes. The midgut gland is responsible for the absorption of small sized particles and nutrients (Ceccaldi, 1989), and is characterized by four cell types: the E- or embryonic from which all cells originate, the R- or resorptive and F- or fibrillar, with both being responsible for assimilation, storage and transportation of nutrients and metals, and the B- or blister cells, responsible for secretion, storage and transport to the intestine (Berillis et al., 2013; Ceccaldi, 1989).

Cadmium in a non-essential trace metal with no known biological function. Levels of cadmium close to coastal areas are known to range from 1-100 ng $L^{-1}$ (Jung and Zauke, 2008; Macken et al., 2009), while in rivers and estuaries values can vary from 0.25-30 µg $L^{-1}$ (Andres et al., 2000; Roast et al., 2001), reaching even higher values due to metal contamination from anthropogenic activities. In its free ionic form, cadmium is considered to be toxic to the organism due to its opportunistic entry in the cell e.g. through the $Ca^{2+}$-transport channels, and for being responsible for the formation of reactive oxygen species (ROS). Cadmium bioaccumulates in the organism due to its strong affinity to sulfur thus

has the tendency to bind to metallothioneins (MTs) and metallothionein-like proteins (MTLP) as a detoxifying defense response.

The aim of the present study was to evaluate the uptake and depuration kinetics of cadmium in the estuarine ditch shrimp *Palaemon varians*, considering three different possible uptake routes: water, diet and a combination of both (water + diet). Within this approach, two versions of the first-order one-compartment toxicokinetic model were used and compared. The rationale supporting the selection of *P. varians* for the present study was based on its: 1) opportunistic feeding habits, as it relies on the consumption of particulate organic matter, benthic micro and macrofauna, as well as zooplankton; 2) eurythermic and euryhaline nature (Morris et al., 2015); 3) wide geographical distribution in European waters; and 4) key role in estuarine food webs (Laffaille et al., 2001) (Sykes et al., 2006). It is also important to highlight that by being an important food item for a number of higher trophic levels (e.g., fish, birds and humans), *P. varians* holds the potential to promote a bottom-up transfer of hazardous chemicals (Seebaugh et al., 2006; 2005).

## 4.3. Materials and Methods

### 4.3.1. Shrimp Culture

Ovigerous females of the ditch shrimp *Palaemon varians* (formerly known as *Palaemonetes varians* (Grave and Ashelby, 2013)) were collected from the end of April to mid-October from a non-polluted salt marsh at Troncalhada, Aveiro, Portugal (40°38'40.1"N, 8°39'52.0"W) (Rodrigues et al., 2011). The organisms were kept in a recirculated maturation system described in detail by Calado et al. (2007) under controlled culture conditions (temperature 20±1 ºC, salinity 35±1, photoperiod 16h light: 08h dark) until larval eclosion (≈2-4 days post collection). Newly hatched larvae (zoea I) were collected and cultured using a recirculated rearing system (Calado et al., 2008) until they had metamorphosed and reached the first postlarval stage (≈12-13 days post hatching). Larvae were fed daily with newly hatched *Artemia* nauplii and decapsulated *Artemia* cysts until used for testing. Postlarvae (3-5 days old) displaying a total dorsal length, measured from the anterior tip of the rostrum to the posterior tip of the telson, of approximately 8.3 ± 0.3 mm and a wet weight of 4.8 ± 0.6 mg were used for the toxicokinetic tests.

### 4.3.2. Test Chemical

The chemical compound used in this study for contaminating both the water, as well as the diet provided (the calanoid copepod *Acartia tonsa*) was cadmium chloride anhydrous (CAS No. 10108-64-2, Sigma-Aldrich, Germany). The concentration of total cadmium selected for the bioconcentration and bioaccumulation tests to contaminate both water and diet was 6.88 µg of Cd $L^{-1}$. The rationale for this option was to simulate the co-existence of predator and prey under the same conditions, therefore deriving realistic estimations of the cadmium accumulation in different levels of a marine trophic chain. Cadmium concentration in the diet at the end of the contamination was 10.5 µg Cd $g^{-1}$ (SD=1.18 µg Cd $g^{-1}$) of copepod's d.w. Additional details related to the bioconcentration and bioaccumulation experiments are further provided in Bioconcentration and Bioaccumulation Tests sections. A stock solution of 100 mg of Cd $L^{-1}$ was prepared using a Millipore® Academic Milli-Q system. Test concentration was then accomplished through dilution in artificial seawater (ASW) (prepared using freshwater purified by a reverse osmosis unit and Tropic Marin® Pro Reef salt; Tropic Marin, Wartenberg, Germany) at a salinity of 35 without changing the final water salinity. Water chemical analysis for cadmium was performed using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Samples from the stock solution and from the concentration tested were acidified after spiking and sent to assess contamination accuracy. The chemical equilibrium model Visual MINTEQ ver. 3.0/3.1 (Gustafsson, 2013) was used to estimate the speciation of cadmium in ASW medium using the concentration of all salt constituents (information supplied by the manufacturer) and the total cadmium concentration to obtain the concentration of cadmium free ion concentration, as well as cadmium free ion activity, which was further used in data analysis. The chemical equilibrium model Visual MINTEQ ver. 3.0/3.1 was allowed to calculate the ionic strength of the test solution, while pH value was fixed to 7.9, temperature was fixed to 20 ºC and salinity was fixed to 35.

### 4.3.3. Bioconcentration Test

A total of 45 *P. varians* postlarvae were exposed to cadmium through contaminated ASW during 96 h (uptake phase) and then transferred to a clean medium (depuration phase) for an identical period of time. Each postlarvae was individually exposed to cadmium in 6-well plate boxes, using 10 mL of volume per each replicate/individual. Postlarvae sampling was performed at 0, 6, 12, 24, 48, 72, 96 h during the uptake phase, and 120, 144, 168

and 192 h for the depuration phase. Each sampling time consisted of three replicates. Shrimp were fed daily after every sampling with non-contaminated dried copepods (*A. tonsa*), consisting of ≈10% of the shrimp's wet body weight (0.45 ± 0.03 mg of dried copepods), with the medium being renewed every day. Copepods were consumed within the initial 20 minutes after providing them as feed to the postlarvae.

### 4.3.4. Bioaccumulation Tests

Copepods provided as diet were previously exposed to 6.88 µg $L^{-1}$ of cadmium, corresponding to the Lowest Observed Effective Concentration obtained in a previous Early Life Stage test by Pavlaki et al. (2016). Copepods were exposed to cadmium in glass aquariums at a density of 100 copepods $L^{-1}$ during 48 h and were then siphoned with the help of a mesh, rinsed with ultrapure water to remove excess of medium, freeze-dried for 24 h and weighed on a microbalance. Each feeding event consisted of ≈10% of the shrimp's wet body weight (0.45 ± 0.03 mg of dried copepods). Cadmium concentration in the diet was 10.5 µg Cd $g^{-1}$ of copepod's dw. For the dietary exposure, a total of 45 *P. varians* postlarvae were exposed to cadmium using contaminated dried copepods (*A. tonsa*), in clean ASW during 96 h (uptake phase), being fed afterwards for an identical period with non contaminated dried copepods (depuration phase). Shrimp were fed daily and after every sampling. For the waterborne + dietary exposure, the same number of shrimp as in the previous tests (45 *P. varians* postlarvae) was used, with specimens being exposed to both cadmium contaminated ASW (same concentration as tested in the bioconcentration tests) and cadmium contaminated copepods during 96 h (uptake phase); following this period, specimens were transferred to clean ASW and fed non-contaminated copepods for another 96 h (depuration phase).

For both tests, cadmium concentration in the diet was 10.5 µg Cd $g^{-1}$ of copepod's dw. Each postlarvae was individually exposed to cadmium, through cadmium-contaminated diet or both cadmium-contaminated water + diet, in 6-well plate boxes, using a volume of 10 mL of exposure media. Sampling was performed at 0, 6, 12, 24, 48, 72, 96 (uptake phase), 120, 144, 168 and 192 h (depuration phase). Every sampling time consisted of three replicates and for every replicate one postlarvae was sampled. During uptake and depuration phase, the ASW was renewed every day.

### 4.3.5. Chemical Analysis

All replicates of *P. varians* were rinsed with ultrapure water to remove excess of medium, freeze-dried for 48 h, weighed using a microbalance and then digested with a mixture of acids, $HNO_3$ and $HClO_4$, at a ratio of 7:1 (v/v, Baker Ultrex II Ultra Pure) using 4 heating steps (step 1: 85 ºC for 60 minutes, step 2: 130 ºC for 60 minutes, step 3: 160 ºC for 60 minutes and step 4: 180 ºC until dryness) in order to destroy all organic material. The residues were taken up in 500 µL of 0.1M $HNO_3$ (Baker Ultrex II Ultra Pure) and cadmium concentration was measured using Graphite Furnace Atomic Absorption Spectrophotometry (Perkin-Elmer PinAAcle 900Z). Three replicate controls and 3 replicates of a certified reference material (CRM) (DOLT-5, Dogfish Liver CRM for trace metals and other constituents) were used to control accuracy of the method (cadmium concentration, 14.5 ± 0.6 mg $kg^{-1}$). Detection limit was 0.112 µg of Cd $L^{-1}$ (n=20). Recovery of cadmium from the certified reference material used for cadmium quantification in the shrimp and copepods was 108%. Values were always calculated and corrected according to the dry weight of the organisms.

### 4.3.6. Toxicokinetic Models

The uptake and depuration kinetics of cadmium in the shrimp were described using a first-order one-compartment model. Two versions of the toxicokinetic model were used to estimate the uptake and depuration rates of cadmium to the organism and compared to derive the best data fit from the three tests. The first version is the classic first-order one-compartment model (**Model 1**). Previous studies performed with *Palaemon* spp. and cadmium have shown that up to 28 days there is no excretion and the metal is generally detoxified in the form of MTs (Rainbow, 2007; Rainbow and White, 1989; White and Rainbow, 1986). Therefore, the second version of the model assumed the existence of an inert fraction (Fi) in the shrimp in which metals can be stored and therefore not contributing to the depuration phase (**Model 2**). Both versions assumed that the background concentration in the shrimp is a fixed value $C_o$ measured in the organism at time zero, which does not take part in the depuration by the kinetics models. **Equations 4.1** and **4.4** represent both models' uptake phase; **Equations 4.2** and **4.5** represent the depuration phase for the first model; and **Equations 4.3** and **4.6** represent the depuration phase for the second model (Tourinho et al., 2015).
For the uptake phase the model used reads:

$$Q(t) = Co + \frac{k_1}{k_2} * C_e * (1 - e^{(-k_2 * t)})$$ (**Equation 4.1**)

For the depuration phase the model used reads:

$$Q(t) = Co + \frac{k_1}{k_2} * C_e * (e^{(-k_2 * (t - t_c))} - e^{(-k_2 * t)})$$ (**Equation 4.2**)

For the depuration phase the model with an inert fraction reads:

$$Q(t) = Co + \frac{k_1}{k_2} * C_e * (Fi + (1 - Fi) * e^{(-k_2 * (t - t_c))})$$ (**Equation 4.3**)

For the worst-case scenario where the organisms are exposed to both contaminated water+diet, the above model equations were adapted to the following:

For the uptake phase the model used reads:

$$Q(t) = Co + \frac{(k_w * C_w + k_f * C_f)}{k_2} * (1 - e^{(-k_2 * t)})$$ (**Equation 4.4**)

For the depuration phase the model used reads:

$$Q(t) = Co + \frac{(k_w * C_w + k_f * C_f)}{k_2} * (e^{(-k_2 * (t - t_c))} - e^{(-k_2 * t)})$$ (**Equation 4.5**)

For the depuration phase the model with the inert fraction reads:

$$Q(t) = Co + \frac{(k_w * C_w + k_f * C_f)}{k_2} * (Fi + (1 - Fi) * e^{(-k_2 * (t - t_c))})$$ (**Equation 4.6**)

,where Q(t) is the concentration in the organism in µg of Cd $g^{-1}$ of dry body weight of the organism at the sampling time t,

$C_0$ is the background concentration in the organism in µg Cd $g^{-1}$ dry body weight at time 0,

$k_1$ is the uptake rate constant in $mL_{water}\ g_{organism}^{-1}\ h^{-1}$ for the test where shrimp were exposed to contaminated water or in $mg_{diet}\ g_{organism}^{-1}\ h^{-1}$ for the test where shrimp were exposed to contaminated diet,

$k_2$ is the depuration rate constant in $h^{-1}$,

$C_e$ is the exposure concentration in the water in µg of $Cd^{2+}\ L^{-1}$ or in the diet in µg of Cd $g^{-1}$,

$k_w$ is the uptake rate constant in $mL_{water}\ g_{organism}^{-1}\ h^{-1}$,

$C_w$ is the exposure concentration in the water in µg of $Cd^{2+}\ L^{-1}$,

$k_f$ is the uptake rate constant in $mg_{diet}\ g_{organism}^{-1}\ h^{-1}$,

$C_f$ is the exposure concentration in the diet in µg of Cd $g^{-1}$,

Fi is the inert fraction in the organism that ranges from 0 to 1(Vijver et al., 2006),

$t_c$ is the time when the organisms where transferred to fresh uncontaminated water in hours, and

t is the sampling time in hours.

Both equations used for the uptake and depuration of cadmium via water or diet by the organism were fitted simultaneously as advised by the OECD guideline 305 (2012). For the estimation of kinetics parameters when the organism was exposed to both

contaminated water + diet, models from individual exposures were fitted simultaneously, with the ones from the worst-case scenario to obtain more accurate k values.

### 4.3.7. Statistical Analysis

The kinetics parameters for every model used were calculated using non-linear regression analysis by fitting the uptake and depuration equation models to the data in the SPSS Statistical Package version 20. An F test was used to compare the models and obtain the best fit to our data (Motulsky and Ransnas, 1987).

Cadmium assimilation efficiency (AE) (α, absorption of cadmium by the shrimp) from the dietary test was estimated by the equation given in the OECD guideline 305 (2012) as:

$\alpha = \frac{C_{0,d} * k_2}{I * C_f} * \frac{1}{1 - e^{-k_2 * t}}$ (**Equation 4.7**)

where, $C_{0,d}$ is the concentration in shrimp at time 0 of the depuration phase in μg of Cd $g^{-1}$ (0.5 μg of Cd $g^{-1}$),

$k_2$ is the depuration rate constant in $h^{-1}$ estimated by **Model 2**,

*I* is the food ingestion rate constant in $g_{diet}\ g_{shrimp}^{-1}\ day^{-1}$ (0.09 $g_{diet}\ g_{shrimp}^{-1}\ day^{-1}$ or 0.004 $g_{diet}\ g_{shrimp}^{-1}\ hour^{-1}$),

$C_f$ is the exposure concentration in the diet in μg of Cd $g^{-1}$ (10.5 μg of Cd $g^{-1}$), and

t is the duration of the feeding period in hours (96 h)

## 4.4. Results

### 4.4.1. Chemical Analysis

Chemical analysis of the stock solution and tested concentration showed a variation of 6% to the nominal concentration (6.88 μg $L^{-1}$) confirming accuracy of the spiking technique.

The Visual MINTEQ equilibrium model estimated the free cadmium ion concentration at 0.31 μg $L^{-1}$ and the cadmium ion activity at 0.09 μg $L^{-1}$, while the dominant cadmium species were $CdCl^+$ (3.60 μg Cd $L^{-1}$) and $CdCl_2^{(aq)}$ (2.97 μg Cd $L^{-1}$).

### 4.4.2. Bioconcentration / Bioaccumulation Tests

During the 8 days of the bioassays no mortality was observed for shrimp in contaminated water or when provided with contaminated diet, while two specimens died when simultaneously exposed to contaminated water + diet. When organisms were exposed to

contaminated diet only one specimen molted, while when exposed to contaminated water or water + diet during the last two days of depuration it was observed that seven and four of the postlarvae molted, respectively. Only living non-molted shrimp were used for the toxicokinetics analysis. In all three exposures, an increase in internal cadmium concentration was observed after the organisms were transferred to the depuration phase and remained constant till the end of the phase.

All calculations presented are based on the concentration of cadmium ion activity calculated using the Visual MINTEQ model. Uptake and depuration kinetics parameters are presented in **Table 4.1** for cadmium ion activity, as described below, while kinetics parameters for total and free ion concentration of cadmium are presented as Supplementary Data (**Table S4.1-S4.2**) for further comparison and use. Depuration rate constant $k_2$ were independent of the type of data used in the model: total cadmium concentration, free ion concentration or cadmium ion activity. In this way, $k_2$ values (and 95% confidence intervals) are similar for the different ways of expressing exposure concentration and only varied with each exposure scenario.

**Table 4.1** Uptake and elimination kinetic parameters for cadmium ions activity in the shrimp *Palaemon varians* under three different exposure scenarios. $C_0$ is the background concentration in the organism in µg Cd $g^{-1}$ dw at time 0, Fi is the inert fraction in the organism, $k_1$ is the uptake rate constant for the aqueous or dietary exposure, $k_2$ is the depuration rate constant, $k_w$ is the uptake rate constant from the aqueous pathway and $k_f$ is the uptake rate constant from the dietary pathway. 95% confidence limits are in brackets, n.d. – not defined.

| **Cd-contaminated medium (ASW) and non-contaminated diet (*A. tonsa*)** | | | | |
|---|---|---|---|---|
| **Model** | **$C_o$ (µg Cd $L^{-1}$)** | **Fi** | **$k_1$ ($mL_{water}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_2$ ($hour^{-1}$)** |
| **1** | 0.23 | - | 126.9 (12.9-241.1) | -0.008 (-) |
| **2** | 0.23 | 0.99 | 285.7 (0-591.04) | 0.01 (0.00-0.03) |
| **Non-contaminated medium (ASW) and Cd-contaminated diet (*A. tonsa*)** | | | | |
| **Model** | **$C_o$ (µg Cd $L^{-1}$)** | **Fi** | **$k_1$ ($mg_{diet}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_2$ ($hour^{-1}$)** |
| **1** | 0.23 | - | 0.22 (0.12-0.32) | -0.005 (n.d.) |
| **2** | 0.23 | 0.99 | 0.30 (0.12-0.47) | 0.007 (0.001-0.014) |
| **Cd-contaminated medium (ASW) and Cd-contaminated diet (*A. tonsa*)** | | | | |
| **Model** | **$C_o$ (µg Cd $L^{-1}$)** | **Fi** | **$k_w$ ($mL_{water}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_f$ ($mg_{diet}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_2$ ($hour^{-1}$)** |
| **1** | 0.24 | - | 235.6 (174.5-296.8) | 0.60 (0.32-0.88) | -0.004 (-) |
| **2** | 0.24 | 0.99 | 377.3 (243.2-511.4) | 0.96 (0.45-1.48) | 0.01 (0.005-0.017) |

Exposure through contaminated water (ASW)

Uptake and Depuration: During the 96 h of uptake, internal cadmium concentration in the shrimp did not reach a steady state phase and increased with time during the uptake phase. Mean internal cadmium concentration in the shrimp was 1.4 µg Cd $g^{-1}$ dw after 96 h of uptake. Depuration rate appears to be slow (**Figure 4.1**).

The kinetics parameters for cadmium ion activity obtained by a first-order one-compartment model, with or without an inert fraction, are presented in **Table 4.1**. **Model 1** estimated a negative depuration rate and hereafter it was set to 0 as negative depurations

cannot be explained biologically. **Model 2** (given by **Equations 4.1** and **4.3**) estimated an inert fraction in the organism of 0.99. Best fit was obtained by **Model 1** ($F_{(1,32)}$=-1.34, n.s.). The fit of both models is displayed in **Figure 4.1**.

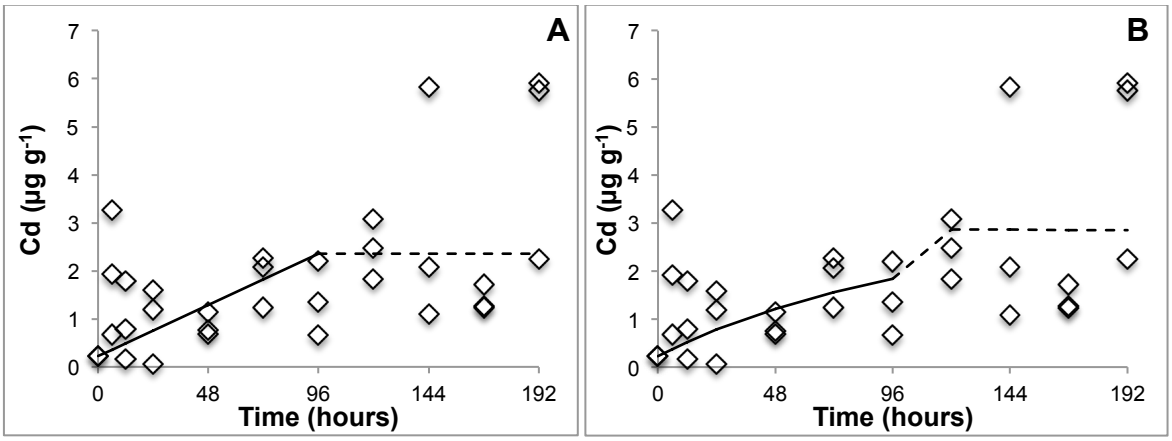


**Figure 4.1** Uptake and depuration kinetics of cadmium ions activity in *Palaemon varians* exposed to contaminated ASW (Artificial Sea Water) using two versions of the one-compartment toxicokinetic model, **A**) classic with no depuration and **B**) with an inert fraction (Fi). Diamond symbol represents real data, the solid line represents data obtained from the uptake model and the dotted line represents data obtained from the depuration model.

Exposure through contaminated diet (*A. tonsa*)

Uptake and Depuration: Internal cadmium concentration showed a slow increase with time during the uptake phase and no steady state was reached after 96 hours of uptake (**Figure 4.2**). Mean internal cadmium concentration in the shrimp was 0.5 µg Cd $g^{-1}$ dw at the end of the uptake phase.

Estimates of the kinetics parameters for cadmium ion activity for both models are presented in **Table 4.1**. Without including the inert fraction in the model, the estimated depuration rate was negative and therefore was set to 0 for further calculations, as biological processes cannot explain a negative depuration rate. **Model 2** estimated an inert fraction of 0.99 in the organism. The best fit was obtained by **Model 1** ($F_{(1,29)}$=1.23, n.s.), with the initial concentration of $C_o$ being fixed to the measured mean value. The fit of both models is displayed in **Figure 4.2**. Cadmium assimilation efficiency (AE) was estimated to be 0.17 (**Equation 4.7**).

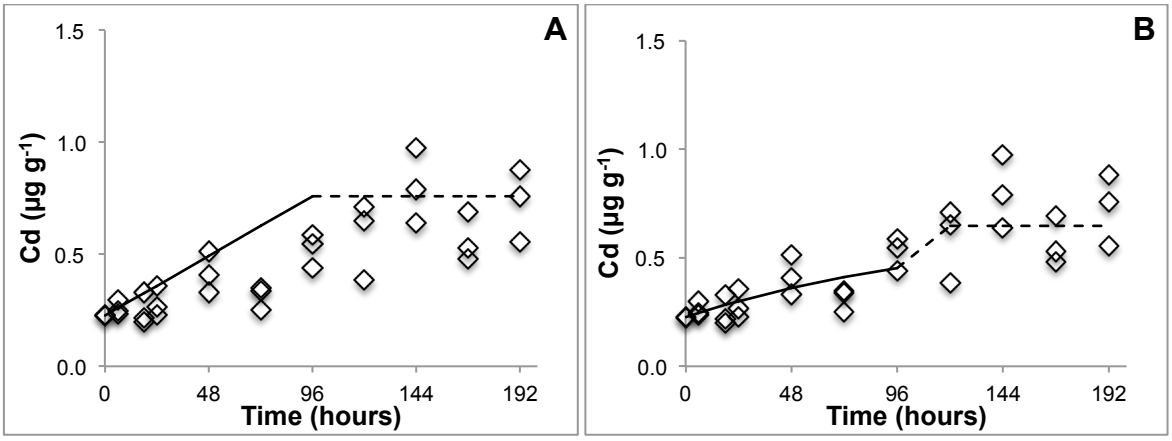


**Figure 4.2** Uptake and depuration kinetics of cadmium ions activity in *Palaemon varians* exposed to contaminated diet, *Acartia tonsa*, using different toxicokinetic models. **A**) classic with no depuration, **B**) with an inert fraction (Fi). Diamond symbol represents real data, the solid line represents data obtained from the uptake model and the dotted line represents data obtained from the depuration model.

Exposure through contaminated water + diet (ASW + *A. tonsa*)

Uptake and Depuration: During the 96 h of uptake, internal cadmium concentration did not reach a steady state phase and increased with time during the uptake phase. Mean internal cadmium concentration in the shrimp was 4.3 µg Cd $g^{-1}$ dw after 96 hours of uptake. Depuration rate appears to be slow. No steady state of internal body concentration was reached during the 96 hours of uptake (**Figure 4.3**).

The kinetics parameters for cadmium ion activity obtained by a first-order one-compartment model with or without an inert fraction are presented in **Table 4.1**. As in the exposure through contaminated diet, the depuration rate constant was set to zero for further calculations. **Model 2** estimated an inert fraction of 0.99 in the organism. A best fit was obtained by **Model 1** ($F_{(1,93)}$=-2.66, n.s.). The fit of both models can be seen in **Figure 4.3**.

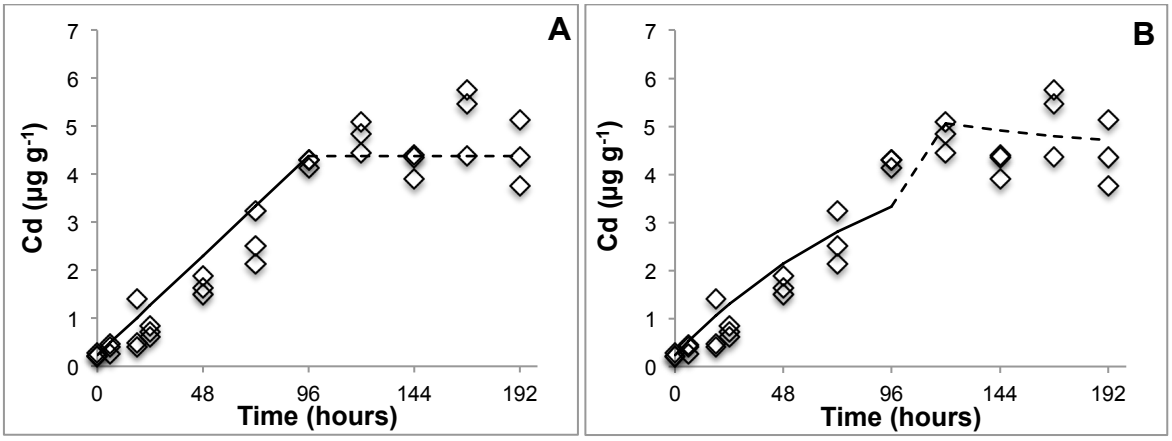


**Figure 4.3** Uptake and depuration kinetics of cadmium ions activity in *Palaemon varians* exposed to both contaminated ASW (Artificial Sea Water) and contaminated diet, *Acartia tonsa*, using different toxicokinetic models. **A**) classic with no depuration, **B**) with an inert fraction (Fi). Diamond symbol represents real data, the solid line represents data obtained from the uptake model and the dotted line represents data obtained from the depuration model.

## 4.5. Discussion

During the experiments carried out in the present study few molting shrimp were observed. In general, crustaceans need to molt to develop and undergo somatic growth (Chang and Mykles, 2011). Molting takes place when ecdysteroid hormones are being secreted by the Y-organ signaling the beginning of a molting cycle (Luo et al., 2015). *Palaemon varians* goes through 5 larval and 5 postlarval phases through molting until it reaches the juvenile stage and each molting cycle lasts approximately two days. Therefore it would be expected that the postlarvae shrimp used in the present study would molt at least twice till the end of the experiment. In this way, the absence of molts during the toxicokinetics bioassays can be associated to an inhibition promoted by cadmium, as this metal is known to prevent the expression of ecdysteroids (Gentile et al., 1982; Rodríguez Moreno et al., 2003).

The increase in the internal cadmium concentration in the shrimp observed during the first day of the depuration phase, and later maintained constant until the end of the experimental trial, can be explained by a possible loss of weight in *P. varians*. Indeed, it was observed a decrease in the mean dry weight of the postlarval shrimp on the first and second day of the depuration phase, however not significant when compared to the last day of uptake (data not shown in the present study).

### 4.5.1. Exposure through contaminated water (ASW)

Shrimp postlarvae show a fast uptake rate when exposed to only contaminated water with no depuration rate being recorded. The lack of cadmium depuration rate recorded for *P. varians* can possibly be explained by the absence of molting events during the experiment. Molting is considered a depuration mechanism *per se*, as it allows the elimination of excess metal body burden due to the opportunistic transfer of $Cd^{2+}$ through $Ca^{2+}$ channels during the exchange of calcium between the hemolymph and the cuticle of the exoskeleton (Bjerregaard and Depledge, 1994; Jung and Zauke, 2008). Jung et al. (2008) reported an internal cadmium concentration in the marine shrimp *Crangon crangon* of approximately 0.7 µg Cd $g^{-1}$, after 4 days of exposure to 5 µg $Cd_{total}$ $L^{-1}$; This value was half the amount measured in the current study and along with a slower uptake rate ($k_1$=31.8 $day^{-1}$ vs 39.8 $day^{-1}$ (or 1.66 $hour^{-1}$), when compared to the uptake rate estimated using total cadmium, please see **Table S4.2** for further comparison). A possible explanation could be the lower temperature the organism was maintained during the test (13 ºC vs. 20 ºC in our study), as lower temperatures are known to slow down several biological processes in shrimp, such as respiration and metabolism (Chang et al., 2009; Gaudy et al., 2000; Howard and Hacker, 1990; Serafim et al., 2002). White and Rainbow (1982) showed that accumulated cadmium in the shrimp *P. elegans* is directly proportional to environmental concentrations and displays values similar to the ones recorded in the present study. Unlike essential metals, cadmium is not regulated by *P. varians* at the concentration used in our study. Other works have also shown experimental evidence that cadmium is not regulated by decapods (Devineau and Triquet, 1985; White and Rainbow, 1986; 1982; Wright, 1977). This is somehow in accordance with the finding of the present study. **Model 1** estimated a negative depuration rate in the shrimp, thus suggesting that cadmium may be stored and not eliminated from the shrimp therefore implying the existence of a stored fraction. **Model 2** estimated a significant percentage of cadmium, almost all of the metal taken up, being stored as an inert fraction in the organism. White and Rainbow (1986) found that *P. elegans* stored cadmium when exposed through water, mainly in the midgut gland, due to the hemolymph transporting cadmium from the gills to the midgut gland and the cuticle, followed by the gills and cuticle. Metian et al. (2010) reported that cadmium in the Pacific blue shrimp, *Litopenaeus stylirostris,* was mainly stored in the cephalothorax and subsequently in the midgut gland in the form of metallothioneins. In order to cope with contaminated environments, estuarine and marine organisms have developed defensive mechanisms such as the production of

metallothionein-like proteins (MTLPs) that sequester and detoxify metals in cells, therefore protecting them from e.g. DNA damage (Howard and Hacker, 1990).

### 4.5.2. Exposure through contaminated diet (*A. tonsa*)

When shrimp postlarvae were exposed to cadmium only through a contaminated diet, uptake rate was low while no depuration was estimated by **Model 1**. **Model 2** estimated that after four days of exposure, the shrimp reached a steady state and almost all of the metal that was being assimilated by the organism was stored and /or detoxified as an inert fraction, while only a small percentage was being eliminated. As mentioned above, the midgut gland is the main organ in which decapods tend to store detoxified metal mainly in the form of MTs. Such high storage percentage would be expected since it is in the midgut that the absorption of nutrients and other dietary products occur due to the existence of mainly R- and F- cells (Berillis et al., 2013). This feature would possibly explain the slow depuration rate recorded in our study. It could initially be expected that cadmium internal concentration and uptake rate would show a faster increase, due to the dissociation of cadmium from the diet during digestion. Consequently, a faster assimilation would also be expected to occur in the midgut and intestinal walls. However, in the present study this assumption was not confirmed. The shrimp's digestive physiology may explain the lower uptake and assimilation of cadmium recorded, as previous studies have shown that *P. varians*' digestive and assimilative activity of cadmium appears to be weaker when compared to other species provided with cadmium contaminated prey (Rainbow et al., 2006; Rainbow and Smith, 2010). The abovementioned studies have also shown that this palaemonid decapod could not assimilate cadmium from three different types of cadmium-contaminated prey that had a subcellular fractionation profile of increased metal rich granules (MRG). Indeed, these authors have shown that cadmium assimilation efficiencies by *P. varians* ranged from 40% to 80%, depending on prey type, with the lowest AE having the highest cadmium distribution percentage in MRG fraction. The low AE of cadmium in shrimp, estimated in the present study to be approximately 20%, could therefore also be due to a higher percentage of MRG in the subcellular fraction of copepods supplied as diet. Barka et al. (2001) reported that the MT fraction (trophically available) measured in copepods, *Tigriopus brevicornis*, after 24 h of exposure to cadmium was higher compared to the MRG fraction, while after 48 h the MT fraction decreased and the MRG fraction increased. Therefore, a plausible conclusion could be that an increase in the MRG content in the copepods would represent a lower trophic

availability of cadmium for the shrimp to digest and assimilate. This scenario would eventually be translated into lower cadmium accumulation, uptake and assimilation rates. Similar results to the ones presented here were found by Boisson et al. (2003), who showed that the uptake and accumulation of waterborne lead to *P. varians* was higher when compared to that of the dietary pathway. The authors refer that their results were supported by the inability of lead to bind to the trophically available fractions and suggested that metal accumulation in the midgut gland was mainly due to water ingestion for osmoregulation.

### 4.5.3. Exposure through contaminated water + diet (ASW + *A. tonsa*)

Most studies focus on the effects and accumulation of cadmium exposure to the organism either through water or diet, while few studies evaluate a scenario that would be more likely to occur in the environment: the exposure to cadmium through both water and diet. Internal cadmium concentration in the shrimp's body increased significantly over time when compared to the concentrations observed for organisms exposed solely to cadmium-contaminated water or a cadmium-contaminated diet. A higher cadmium accumulation can be expected if the organism has two pathways for cadmium uptake: through water and through diet. **Model 2** provided the existence of an inert fraction, which once again seems to be in accordance with **Model 1**, by estimating a negative depuration rate in the shrimp, suggests that cadmium may be stored in the organism and not excreted. As suggested by previous studies, cadmium tends to be stored and detoxified, mainly due to the production of metallotheioneins (MTs) (Amiard et al., 2006; Klaassen et al., 1999) and as already mentioned, decapod crustaceans accumulate and store excess of metal in the midgut gland due to the existence of certain types of cells.

Even though the two ways of exposure tested in the present work were clearly distinct, the addition of contaminated food to contaminated water to simulate a worst-case scenario may have promoted an increase in the cadmium content of copepods. It should not be discarded that this effect may have enhanced the uptake of cadmium by *P. varians* through diet. While copepods were offered in a freeze-dried form and consequently no cadmium uptake could take place from the water, however, cadmium could still adsorb to the copepod's outer surface. Metal binding from the water to the diet has already been highlighted by several authors (Jung and Zauke, 2008; Williams et al., 2010), as it can facilitate the assimilation of metals in the midgut gland during digestion.

**Model 1** estimated that no steady state is reached under diet and water + diet exposure

routes as the internal concentration will keep increasing and therefore it is uncertain whether biomagnification may occur, as it will depend on the critical body residue (CBR) of the organism that will lead to mortality. If the critical body residue in the organism is lower than the concentration in the prey then no biomagnification occurs.

### 4.5.4. Comparison between exposures

In order to be able to compare the shrimp performance between exposures, the one-compartment model without an inert fraction was used due to its best fit to the data from all three exposures. As **Model 1** estimated a negative cadmium depuration rate in all exposure routes, this would indicate that all accumulated cadmium is not eliminated from the shrimp's body thus suggesting the existence of an inert fraction equal to the unity. Even though **Model 2** did not show an improvement of the data fit, it was also able to quantify and support such theory. Such assumption can be based on previous studies on the accumulation patterns of cadmium by decapod crustaceans. As an example one can refer to the study by Rainbow and White (Rainbow and White, 1989) where the authors refer that *Palaemon elegans* showed a slow rate of cadmium uptake while no elimination was observed during 28 days of exposure through water, suggesting that all metal was stored. White and Rainbow (White and Rainbow, 1982) demonstrated that cadmium is accumulated and not regulated by *P. elegans*, thus reinforcing the idea that cadmium may promote such a high accumulated fraction.

Exposure to cadmium through contaminated water + diet promoted a higher uptake rate followed by exposure through water, then through diet alone. Such result would be somehow expected, as already referred above, due to the two uptake pathways: water and diet. The higher accumulation rates recorded when *P. varians* is exposed to contaminated water or contaminated water + diet are promoted by the direct contact of the metal and the gills and the transfer of cadmium from the gills to the midgut gland via the hemolymph (Bambang et al., 1995); along with the faster assimilation rate to the midgut gland by the digestive system, when compared to the uptake through diet alone (White and Rainbow, 1986). Uptake rates of cadmium, either through water or through diet, seemed to be enhanced when the shrimp is exposed to both pathways simultaneously, possibly indicating an additive or even a synergistic effect.

Depuration rates recorded for all three exposures, waterborne, dietary and worst-case scenario (waterborne + dietary), were negative. Such results could indicate that the way of exposure did not play a key role in cadmium depuration for *P. varians* postlarvae.

## 4.6. Conclusions

The estuarine shrimp *P. varians* appears to display a high capacity of accumulating cadmium under each of the exposure scenarios tested in the present work: through water, diet or water + diet. However, when looking at exposure through diet, one can conclude that total cadmium concentration in the prey should not be used as a standard to understand biomagnification. Instead, the trophically available metal fraction (TAM) should be used and further studies on the subcellular fractionation of cadmium in prey are required to better understand these pathways. Both versions of the one-compartment model used in the present study indicated the potential existence of a fraction that is stored in the organism, which may cause metal concentrations to accumulate above the organisms' threshold and eventually lead to mortality. Moreover, the increased metal capacity of *P. varian*s and the lack of depuration could pose a potential threat to trophic webs, as this species is an important link to higher trophic levels, such as fish, birds and humans.

## 4.7. References

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat Toxicol 76, 160–202. doi:10.1016/j.aquatox.2005.08.015

Andres, S., Ribeyre, F., Tourencq, J.N., Boudou, A., 2000. Interspecific comparison of cadmium and zinc contamination in the organs of four fish species along a polymetallic pollution gradient (Lot River, France). Sci Total Environ 248, 11–25. doi:10.1016/S0048-9697(99)00477-5

Bambang, Y., Charmantier, G., Thuet, P., Trilles, J.P., 1995. Effect of cadmium on survival and osmoregulation of various developmental stages of the shrimp *Penaeus japonicus* (Crustacea: Decapoda). Mar. Biol. 123, 443–450. doi:10.1007/BF00349223

Barka, S., Pavillon, J.F., Amiard, J.C., 2001. Influence of different essential and non-essential metals on MTLP levels in the Copepod *Tigriopus brevicornis*. Comp Biochem Physiol C 128, 479–493. doi:10.1016/S1532-0456(00)00198-8

Berillis, P., Simon, C., Mente, E., Sofos, F., Karapanagiotidis, I.T., 2013. A novel image processing method to determine the nutritional condition of lobsters. Micron 45, 140–144. doi:10.1016/j.micron.2012.10.014

Bjerregaard, P., Depledge, M.H., 1994. Cadmium accumulation in *Littorina littorea*, *Mytilus edulis* and *Carcinus maenas*: the influence of salinity and calcium ion concentrations. Mar. Biol. 119, 385–395. doi:10.1007/BF00347535

Boisson, F., Cotret, O., Teyssié, J.L., El-Baradeï, M., Fowler, S.W., 2003. Relative importance of dissolved and food pathways for lead contamination in shrimp. Marine Poll Bull 46, 1549–1557. doi:10.1016/S0025-326X(03)00316-3

Bolton-Warberg, M., Coen, L.D., Weinstein, J.E., 2006. Acute Toxicity and Acetylcholinesterase Inhibition in Grass Shrimp (*Palaemonetes pugio*) and Oysters (*Crassostrea virginica*) Exposed to the Organophosphate Dichlorvos: Laboratory and Field Studies. Arch Environ Contam Toxicol 52, 207–216. doi:10.1007/s00244-005-0325-z

Calado, R., Pimentel, T., Vitorino, A., Dionísio, G., Dinis, M.T., 2008. Technical improvements of a rearing system for the culture of decapod crustacean larvae, with emphasis on marine ornamental species. Aquaculture 285, 264–269. doi:10.1016/j.aquaculture.2008.08.019

Calado, R., Vitorino, A., Dionísio, G., Dinis, M.T., 2007. A recirculated maturation system for marine ornamental decapods. Aquaculture 263, 68–74.

doi:10.1016/j.aquaculture.2006.10.013

Ceccaldi, H., 1989. Anatomy and physiology of digestive tract of Crustaceans Decapods reared in aquaculture. Advances in Tropical Aquaculture.

Chang, E.S., Mykles, D.L., 2011. Regulation of crustacean molting: a review and our perspectives. Gen. Comp. Endocrinol. 172, 323–330. doi:10.1016/j.ygcen.2011.04.003

Chang, M., Wang, W.-N., Wang, A.-L., Tian, T.-T., Wang, P., Zheng, Y., Liu, Y., 2009. Effects of cadmium on respiratory burst, intracellular $Ca^{2+}$ and DNA damage in the white shrimp *Litopenaeus vannamei*. Comp Biochem Physiol C 149, 581–586. doi:10.1016/j.cbpc.2008.12.011

Devineau, J., Triquet, C.A., 1985. Patterns of Bioaccumulation of an Essential Trace-Element (Zinc) and a Pollutant Metal (Cadmium) in Larvae of the Prawn *Palaemon serratus*. Mar. Biol. 86, 139–143.

Gaudy, R., Cervetto, G., Pagano, M., 2000. Comparison of the metabolism of *Acartia clausi* and *A. tonsa*: influence of temperature and salinity. J Exp Mar Biol Ecol 247, 51–65.

Gentile, S.M., Gentile, J.H., Walker, J., Heltshe, J.F., 1982. Chronic effects of cadmium on two species of mysid shrimp: *Mysidopsis bahia* and *Mysidopsis bigelowi*, in: Ecology of Mysidacea. Springer Netherlands, Dordrecht, pp. 195–204. doi:10.1007/978-94-009-8012-9_21

Goetze, S., Matoo, O.B., Beniash, E., Saborowski, R., Sokolova, I.M., 2014. Interactive effects of $CO_2$ and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. Aquat Toxicol 149, 65–82. doi:10.1016/j.aquatox.2014.01.027

Grave, S.D., Ashelby, C.W., 2013. A re-appraisal of the systematic status of selected genera in Palaemoninae (Crustacea: Decapoda: Palaemonidae). Zootaxa 3734, 331. doi:10.11646/zootaxa.3734.3.3

Gustafsson, J.P., 2013. Visual MINTEQ ver. 3.0/3.1 [WWW Document]. Visual MINTEQ - A free equilibrium speciation model. URL http://vminteq.lwr.kth.se/ (accessed 9.9.15).

Howard, C.L., Hacker, C.S., 1990. Effects of Salinity, Temperature, and Cadmium on Cadmium-Binding Protein in the Grass Shrimp, *Palaemonetes pugio*. Arch Environ Contam Toxicol 19, 341–347. doi:10.1007/BF01054976

Isani, G., Andreani, G., Cocchioni, F., Fedeli, D., Carpené, E., Falcioni, G., 2009. Cadmium accumulation and biochemical responses in *Sparus aurata* following sub-lethal Cd exposure. Ecotoxicol Environ Safety 72, 224–230.

doi:10.1016/j.ecoenv.2008.04.015

Jung, K., Zauke, G.P., 2008. Bioaccumulation of trace metals in the brown shrimp Crangon crangon (Linnaeus, 1758) from the German Wadden Sea. Aquat Toxicol 88, 243–249. doi:10.1016/j.aquatox.2008.05.007

Kaoud, H.A., Eldahshan, A.R., 2010. Bioaccumulation of cadmium in the freshwater prawn *Macrobrachium rosenbergii*. Nature and Science.

Klaassen, C.D., Liu, J., Choudhuri, S., 1999. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu. Rev. Pharmacol. Toxicol. 39, 267–294. doi:10.1146/annurev.pharmtox.39.1.267

Laffaille, P., Lefeuvre, J.C., Schricke, M.T., Feunteun, E., 2001. Feeding ecology of o-group sea bass, *Dicentrarchus labrax*, in salt marshes of Mont Saint Michel Bay (France). Estuaries 24, 116–125. doi:10.2307/1352818

Luo, J., Pei, S., Jing, W., Zou, E., Wang, L., 2015. Cadmium inhibits molting of the freshwater crab *Sinopotamon henanense* by reducing the hemolymph ecdysteroid content and the activities of chitinase and N-acetyl-β-glucosaminidase in the epidermis. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 169, 1–6. doi:10.1016/j.cbpc.2014.10.003

Macken, A., Giltrap, M., Ryall, K., Foley, B., McGovern, E., McHugh, B., Davoren, M., 2009. A test battery approach to the ecotoxicological evaluation of cadmium and copper employing a battery of marine bioassays. Ecotoxicology 18, 470–480. doi:10.1007/s10646-009-0305-6

Metian, M., Hédouin, L., Eltayeb, M.M., Lacoue-Labarthe, T., Teyssié, J.-L., Mugnier, C., Bustamante, P., Warnau, M., 2010. Metal and metalloid bioaccumulation in the Pacific blue shrimp *Litopenaeus stylirostris* (Stimpson) from New Caledonia: Laboratory and field studies. Marine Poll Bull 61, 576–584. doi:10.1016/j.marpolbul.2010.06.035

Morcillo, P., Esteban, M.A., Cuesta, A., 2016. Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. Chemosphere 144, 225–233. doi:10.1016/j.chemosphere.2015.08.020

Morris, J.P., Thatje, S., Ravaux, J., Shillito, B., Hauton, C., 2015. Characterising multi-level effects of acute pressure exposure on a shallow-water invertebrate: insights into the kinetics and hierarchy of the stress response. J Exp Biol 218, 2594–2602. doi:10.1242/jeb.125914

Motulsky, H.J., Ransnas, L.A., 1987. Fitting curves to data using nonlinear regression: a practical and nonmathematical review. FASEB J 1, 365–374. doi:10.1096/fj.1530-6860

OECD, 2012. OECD Guidelines for the Testing of Chemicals, Section 3: Degradation and Accumulation Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure. OECD Publishing. doi:10.1787/9789264185296-en

Pavlaki, M.D., Araújo, M.J., Cardoso, D.N., Silva, A.R.R., Cruz, A., Mendo, S., Soares, A.M.V.M., Calado, R., Loureiro, S., 2016. Ecotoxicity and genotoxicity of cadmium in different marine trophic levels. Environ Pollut 215, 203–212. doi:10.1016/j.envpol.2016.05.010

Rainbow, P.S., 2007. Trace metal bioaccumulation: models, metabolic availability and toxicity. Environ Int 33, 576–582. doi:10.1016/j.envint.2006.05.007

Rainbow, P.S., Poirier, L., Smith, B.D., Brix, K.V., Luoma, S.N., 2006. Trophic transfer of trace metals from the polychaete worm *Nereis diversicolor* to the polychaete *N. virens* and the decapod crustacean *Palaemonetes varians*. Mar. Ecol. Prog. Ser. 321, 167–181. doi:10.3354/meps321167

Rainbow, P.S., Smith, B.D., 2010. Trophic transfer of trace metals: Subcellular compartmentalisation in bivalve prey and comparative assimilation efficiencies of two invertebrate predators. J Exp Mar Biol Ecol 390, 143–148. doi:10.1016/j.jembe.2010.05.002

Rainbow, P.S., White, S.L., 1989. Comparative strategies of heavy metal accumulation by crustaceans: zinc, copper and cadmium in a decapod, an amphipod and a barnacle. Hydrobiologia 174, 245–262. doi:10.1007/BF00008164

Rajkumar, J.S., John Milton, M.C., Ulthiralingam, M., Azhaguraj, R., Ganesh, J., Ambrose, T., 2011. Toxic effects and bioaccumulation of cadmium, copper, lead and zinc in post larval stages of *Penaeus monodon*. International Journal of Development Research 1, 1–5. doi:2230-9926

Roast, S.D., Widdows, J., Jones, M.B., 2001. Effects of salinity and chemical speciation on cadmium accumulation and toxicity to two mysid species. Environ Toxicol Chem 20, 1078–1084. doi:10.1002/etc.5620200519

Rocha, T.L., Gomes, T., Pinheiro, J.P., Sousa, V.S., Nunes, L.M., Teixeira, M.R., Bebianno, M.J., 2015a. Toxicokinetics and tissue distribution of cadmium-based Quantum Dots in the marine mussel *Mytilus galloprovincialis*. Environ Pollut 204, 207–214. doi:10.1016/j.envpol.2015.05.008

Rocha, T.L., Gomes, T., Sousa, V.S., Mestre, N.C., Bebianno, M.J., 2015b. Ecotoxicological impact of engineered nanomaterials in bivalve molluscs: An overview. Mar Environ Res 111, 74–88. doi:10.1016/j.marenvres.2015.06.013

Rodrigues, C.M., Bio, A., Amat, F., Vieira, N., 2011. Artisanal salt production in

Aveiro/Portugal - an ecofriendly process. Saline Syst. 7, 3. doi:10.1186/1746-1448-7-3

Rodríguez Moreno, P.A., Medesani, D.A., Rodríguez, E.M., 2003. Inhibition of molting by cadmium in the crab *Chasmagnathus granulata* (Decapoda Brachyura). Aquat Toxicol 64, 155–164. doi:10.1016/S0166-445X(03)00029-8

Seebaugh, D.R., Estephan, A., Wallace, W.G., 2006. Relationship Between Dietary Cadmium Absorption by Grass Shrimp (*Palaemonetes pugio*) and Trophically Available Cadmium in Amphipod (*Gammarus lawrencianus*) Prey. Bull Environ Contam Toxicol 76, 16–23. doi:10.1007/s00128-005-0884-8

Seebaugh, D.R., Goto, D., Wallace, W.G., 2005. Bioenhancement of cadmium transfer along a multi-level food chain. Mar Environ Res 59, 473–491. doi:10.1016/j.marenvres.2004.07.003

Serafim, M.A., Company, R.M., Bebianno, M.J., Langston, W.J., 2002. Effect of temperature and size on metallothionein synthesis in the gill of *Mytilus galloprovincialis* exposed to cadmium. Mar Environ Res 54, 361–365. doi:10.1016/S0141-1136(02)00121-6

Souid, G., Souayed, N., Yaktiti, F., Maaroufi, K., 2013. Effect of acute cadmium exposure on metal accumulation and oxidative stress biomarkers of *Sparus aurata*. Ecotoxicol Environ Safety 89, 1–7. doi:10.1016/j.ecoenv.2012.12.015

Sykes, A.V., Domingues, P.M., Andrade, J.P., 2006. Effects of Using Live Grass Shrimp (*Palaemonetes varians*) as the only Source of Food for the Culture of Cuttlefish, *Sepia officinalis* (Linnaeus, 1758). Aquaculture International 14, 551–568. doi:10.1007/s10499-006-9054-1

Tan, Q.-G., Wang, W.-X., 2012. Two-Compartment Toxicokinetic–Toxicodynamic Model to Predict Metal Toxicity in *Daphnia magna*. Environ. Sci. Technol. 46, 9709–9715. doi:10.1021/es301987u

Tourinho, P.S., van Gestel, C.A.M., Morgan, A.J., Kille, P., Svendsen, C., Jurkschat, K., Mosselmans, J.F.W., Soares, A.M.V.M., Loureiro, S., 2015. Toxicokinetics of Ag in the terrestrial isopod *Porcellionides pruinosus* exposed to Ag NPs and $AgNO_3$ via soil and food. Ecotoxicology 1–12. doi:10.1007/s10646-015-1585-7

Viarengo, A., Canesi, L., 1991. Mussels as biological indicators of pollution. Aquaculture 94, 225–243. doi:10.1016/0044-8486(91)90120-V

Vijver, M.G., Vink, J.P.M., Jager, T., van Straalen, N.M., Wolterbeek, H.T., van Gestel, C.A.M., 2006. Kinetics of Zn and Cd accumulation in the isopod *Porcellio scaber* exposed to contaminated soil and/or food. Soil Biol Biochem 38, 1554–1563.

doi:10.1016/j.soilbio.2005.11.006

White, S.L., Rainbow, P.S., 1986. Accumulation of cadmium by *Palaemon elegans* (Crustacea: Decapoda). Mar. Ecol. Prog. Ser.

White, S.L., Rainbow, P.S., 1982. Regulation and Accumulation of Copper, Zinc and Cadmium by the Shrimp *Palaemon elegans*. Mar. Ecol. Prog. Ser. 8, 95–101.

Williams, J.J., Dutton, J., Chen, C.Y., Fisher, N.S., 2010. Metal (As, Cd, Hg, and CH3Hg) bioaccumulation from water and food by the benthic amphipod *Leptocheirus plumulosus*. Environ Toxicol Chem 29, 1755–1761. doi:10.1002/etc.207

Wright, D.A., 1977. The Effect of Salinity on Cadmium Uptake by the Tissues of the Shore Crab *Carcinus Maenas*. J Exp Biol 67, 137–146.

## 4.8. Supplementary Data

**Table S4.1** Uptake and elimination kinetic parameters for free cadmium ions concentration in the shrimp *Palaemon varians* under three different exposure conditions. $C_0$ is the background concentration in the organism in µg Cd $g^{-1}$ dw at time 0, Fi is the inert fraction in the organism, $k_1$ is the uptake rate constant for the aqueous or dietary exposure, $k_2$ is the depuration rate constant, $k_w$ is the uptake rate constant from the aqueous pathway and $k_f$ is the uptake rate constant from the dietary pathway. 95% confidence limits are in brackets, n.d. – not defined.

| **Cd-contaminated ASW and non-contaminated diet (*A. tonsa*)** | | | | |
|---|---|---|---|---|
| **Model** | **$C_o$ (µg Cd $L^{-1}$)** | **Fi** | **$k_1$ ($mL_{water}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_2$ ($hour^{-1}$)** |
| **1** | 0.23 | - | 36.99 (3.76-70.21) | -0.008 (-) |
| **2** | 0.23 | 0.99 | 83.21 (0-172.18) | 0.01 (0-0.026) |
| **Non-contaminated ASW and Cd-contaminated diet (*A. tonsa*)** | | | | |
| **Model** | **$C_o$ (µg Cd $L^{-1}$)** | **Fi** | **$k_1$ ($mg_{diet}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_2$ ($hour^{-1}$)** |
| **1** | 0.23 | - | 0.22 (0.12-0.32) | -0.005 (-) |
| **2** | 0.23 | 0.99 | 0.30 (0.12-0.47) | 0.007 (0.001-0.014) |

| **Cd-contaminated ASW and Cd-contaminated diet (*A. tonsa*)** | | | | | |
|---|---|---|---|---|---|
| **Model** | **$C_o$ (µg Cd $L^{-1}$)** | **Fi** | **$k_m$ ($mL_{water}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_f$ ($mg_{diet}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_2$ ($hour^{-1}$)** |
| **1** | 0.24 | - | 68.63 (50.84-86.43) | 0.60 (0.32-0.88) | -0.004 (-) |
| **2** | 0.24 | 0.99 | 109.9 (70.9-149.0) | 0.96 (0.45-1.48) | 0.01 (0.05-0.017) |

**Table S4.2** Uptake and elimination kinetic parameters for total cadmium concentration in the shrimp *Palaemon varians* under three different exposure conditions. $C_0$ is the background concentration in the organism in µg Cd $g^{-1}$ dw at time 0, Fi is the inert fraction in the organism, $k_1$ is the uptake rate constant for the aqueous or dietary exposure, $k_2$ is the depuration rate constant, $k_w$ is the uptake rate constant from the aqueous pathway, $k_f$ is the uptake rate constant from the dietary pathway, BCF is the bioconcentration factor, BMF is the biomagnification factor and $DT_{50}$ is the time needed for the organisms to eliminate half the amount of cadmium. 95% confidence intervals are in brackets, n.d. – not defined.

| **Cd-contaminated ASW and non-contaminated diet (*A. tonsa*)** | | | | |
|---|---|---|---|---|
| **Model** | **$C_o$ (µg Cd $L^{-1}$)** | **Fi** | **$k_1$ ($mL_{water}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_2$ ($hour^{-1}$)** |
| **1** | 0.23 | - | 1.66 (0.17-3.15) | -0.008 (-) |
| **2** | 0.23 | 0.99 | 3.74 (0-7.73) | 0.01 (0-0.03) |
| **Non-contaminated ASW and Cd-contaminated diet (*A. tonsa*)** | | | | |
| **Model** | **$C_o$ (µg Cd $L^{-1}$)** | **Fi** | **$k_1$ ($mg_{diet}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_2$ ($hour^{-1}$)** |
| **1** | 0.23 | - | 0.22 (0.12-0.32) | -0.005 (-) |
| **2** | 0.23 | 0.99 | 0.30 (0.12-0.47) | 0.007 (0.001-0.014) |

| **Cd-contaminated ASW and Cd-contaminated diet (*A. tonsa*)** | | | | | |
|---|---|---|---|---|---|
| **Model** | **$C_o$ (µg Cd $L^{-1}$)** | **Fi** | **$k_m$ ($mL_{water}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_f$ ($mg_{diet}$ $g_{organism}^{-1}$, $hour^{-1}$)** | **$k_2$ ($hour^{-1}$)** |
| **1** | 0.24 | - | 3.08 (2.28-3.88) | 0.60 (0.32-0.88) | -0.004 (-) |
| **2** | 0.24 | 0.99 | 4.94 (3.18-6.69) | 0.96 (0.45-1.48) | 0.01 (0.005-0.017) |

# Chapter 5

# Cadmium kinetics and distribution in *Solea senegalensis* tissues under dietary and water exposure and the link to human health

Submitted

# Cadmium kinetics and distribution in *Solea senegalensis* tissues under dietary and water exposure and the link to human health

## 5.1. Abstract

Bioaccumulation of cadmium was assessed in the different tissues of the benthic fish *Solea senegalensis*. Senegalese soles were exposed to cadmium-contaminated diet and water during 14 days and following this period were let to depurate in clean water and non-contaminated diet for another 14 days. Cadmium content was measured in four different tissues, muscle, gills, liver and intestine, with recorded values increasing from the muscle to gills, followed by the liver, with the highest values being recorded in the intestine. Muscle tissue showed a considerably low cadmium accumulation over 14 days of uptake. According to cadmium's kinetics in the sole, the highest uptake flux of the metal was observed in the intestine, likely resulting from the direct contact and assimilation of the metal from the contaminated diet (the ragworm *Hediste diversicolor*) and the high drinking rate exhibited by marine fish due to the hypertonic environment they live in. Cadmium depuration rate from the liver was nonexistent, thus implying the existence of a storage compartment for the metal during uptake and depuration. The second lowest depuration rate was observed in the gills, in which cadmium concentration showed relatively no changes. This finding confirms that this metal can be accumulated and stored in chloride cells due to its high affinity and similarity to $Ca^{2+}$, the most common pathway for cadmium to enter the cellular membranes. Additionally, cadmium may also be redistributed back to the gills through the circulatory system, in order to be excreted with excess of salts to maintain the fish osmotic balance. Comparisons between the maximum acceptable values for cadmium in the muscle, THQ and EWI show that the cadmium measured in the muscle of juvenile soles from the present study is within acceptable limits and can be considered safe for human consumption.

Keywords: benthic fish, uptake flux, depuration, *Hediste diversicolor*, metal, toxicokinetics

## 5.2. Introduction

Estuarine ecosystems are dynamic environments located on the transition zone between marine and freshwater habitats and widely considered of high ecological and economic value (Fonseca et al., 2011 and references therein; Goetze et al., 2014). Such interface position makes these ecosystems highly susceptible to drastic fluctuations on environmental conditions, either due to environmental or anthropogenic activities (Goetze et al., 2014). The accumulation of metals in estuaries has long been considered as a significant threat to their biodiversity (Creighton and Twining, 2010). Even though metals occur naturally in the environment, as a result of environmental processes, the increase of anthropogenic activities close to coastal areas have enhanced the input of metals to estuarine and marine ecosystems (Barhoumi et al., 2009). While certain metals, such as copper and zinc, are considered essential, others like cadmium or lead are until now considered non-essential to biological processes and therefore, even when present at low concentrations, they may constitute a potential threat to aquatic organisms (Singh et al., 2011; Tchounwou et al., 2012). A considerable number of marine species, some of which of high socio-economic value, tend to use estuarine ecosystems as nursery grounds (Cabral and Costa, 1999; Fonseca et al., 2015) and therefore, have been used in several studies as biological indicators of habitat quality (Costa et al., 2008; Fonseca et al., 2015; 2011; Galindo et al., 2012). When the lower levels of an aquatic trophic web are exposed to metals, bioaccumulation is likely to occur and, depending on the metal-organism interactions (e.g., ways of uptake, storage, detoxification and elimination), these compounds can be transferred bottom-up along the trophic chain towards higher trophic levels, such as fish, and eventually reach humans. As fish play a key role in marine/estuarine ecosystems and are used for human consumption, their survey in terms of metal contamination is paramount for environmental and human health (Mathews and Fisher, 2009). Organisms accumulate the biologically available forms of metals, thus enabling the continuous monitoring of pollutants (Barhoumi et al., 2009). Accordingly, Munger et al. (1997 and references therein) have highlighted the concentration of free metal ion activity as a more accurate way to predict bioaccumulation in the organisms.

Bioaccumulation is commonly used as a general term to describe the level of concentration of a given chemical compound in an organism in relation to the external exposure concentration (dietary, dermal, etc.) (Gobas and Morrison, 2000). However, the term is usually used to describe the process occurring under field conditions. The terms bioconcentration and biomagnification are in fact more accurate when aiming to describe

the process of chemical concentration in an organism as a result of waterborne or dietary exposure, respectively (Gobas and Morrison, 2000). Although the toxic effects and patterns of cadmium accumulation have been assessed at low trophic levels using several phytoplanktonic (Leborans and Novillo, 1996; Sisman-Aydin et al., 2013; Wang and Wang, 2009; 2008) and zooplanktonic organisms (Bjerregaard and Depledge, 1994; Jung and Zauke, 2008; Keating et al., 2007; Metian et al., 2010; Xu et al., 2001), strict ethic constraints often discourage the use of higher trophic levels, such as fish. Besides, the scarce number of studies assessing cadmium accumulation in marine vertebrates generally solely consider a single route of exposure, either through water or diet (Burgos and Rainbow, 2001; Cao et al., 2012; Kim et al., 2006; 2004). However, considering that in natural environments marine organisms are often simultaneously exposed to multiple contaminated compartments, the assessment of multiple routes of exposure is an important step towards a more realistic understanding of cadmium accumulation patterns, not only in the organism itself but also within and between different links of marine/estuarine trophic webs. Single-route approaches may not only lead to underestimations of total metal accumulation but also to a biased perception of metal distribution in internal tissues, both being highly relevant from an ecosystem and human health perspective.

Fish bioaccumulation experiments can be particularly important for benthic species destined for human consumption, as these live in close contact with metal-contaminated sediments and feed upon sediment-dwelling macroinvertebrates highly prone to the dermal uptake of metals (Burgos and Rainbow, 2001). Furthermore, these fish species often constitute relevant components of human diets so they can easily become an important pathway for human exposure to metals.

The present study aimed to assess: i) the bioaccumulation patterns of cadmium in the Senegalese sole, *Solea senegalensis* Kaup, 1858, a species present in the coastal waters of the Mediterranean and eastern Atlantic (Eichinger et al., 2010), which is widely used for human consumption, targeted by commercial fisheries and cultured in earth ponds and recirculated systems in fish farms (Dinis et al., 1999; Imsland et al., 2003); and ii) its possible risks and implications to human health. In order to achieve the first objective, we simultaneously exposed Senegalese soles to waterborne and dietary cadmium (using an estuarine benthic organism, the ragworm, *Hediste diversicolor*) for an uptake period of 14 days followed by another 14 days of depuration. Fish were then sampled at different time points during uptake and depuration for cadmium content measurements in different tissues (muscle, gills, liver and intestine). Concerning the second objective, a non-

carcinogenic Target Hazard Quotient (THQ) established by the US Environmental Protection Agency (United States Environmental Protection Agency (US EPA), 2010) was used, as well as the Estimated Weekly Intake (EWI) to evaluate the potential risks of cadmium to human health originating from the consumption of Senegalese sole.

## 5.3. Materials and Methods

Animal experimentation was authorized by the local welfare ethics committee and followed the Portuguese law for animal experimentation (Regulatory Guideline nº 1005/92, October 23rd, 1992).

### 5.3.1. Sole Stock

A total of 32 juvenile *Solea senegalensis* (with an average (± S.D.) wet weight of 17.77 ± 2.04 g and total length of 11.88 ± 0.68 cm) were kindly provided by Safistela – a commercial Senegalese sole hatchery from group Sea8 located in Póvoa de Varzim, Portugal. Juvenile Senegalese soles were transferred to the University of Aveiro, Department of Biology, inside plastic bags under constant temperature and in the dark, followed by their acclimatization in the laboratory. Soles were maintained during acclimatization (14 days) and experimentation (28 days) in a Recirculated Modular System (RMS), as described in the Basic Set-up of the Recirculated Modular System (RMS) section and shown in **Figure 5.1**.

### 5.3.2. Polychaete Stock

Ragworms *Hediste diversicolor* were sampled by hand during low tide from a low-contaminated site area at Praia da Barra, Aveiro, Portugal (40°38'13.3"N 8°44'29.4"W) (Figueira et al., 2012; Velez et al., 2015). Organisms were kept in artificial seawater (ASW), previously prepared by mixing Tropic Marin Pro Reef Salt (Tropic Marin® Pro Reef, Germany) with water purified by reverse osmosis (Aqua-win RO-6080, Thailand) at a salinity 35 and temperature of 18 ºC. During the acclimatization period (4 days), *H. diversicolor* were kept in clean ASW and fed *ad libitum* with TetraMin® fish flakes. This procedure aimed at replacing their digestive tract content before providing the polychaetes as diet to soles (Geffard et al., 2005). After this period, polychaetes were

contaminated with cadmium for the uptake phase or supplied directly as non-contaminated diet during the depuration period.

### 5.3.3. Test Chemical

The chemical compound used in this study was cadmium chloride anhydrous (CAS No. 10108-64-2, Sigma-Aldrich, Germany). A stock solution of 1 g of Cd $L^{-1}$ was prepared using a Millipore® Academic Milli-Q system. The tested concentration of 6.88 µg of Cd $L^{-1}$ at salinity 35 was then accomplished through dilution without changing the final water salinity in previously prepared ASW. The non-lethal total cadmium concentration of 6.88 µg $L^{-1}$ for both water and diet contamination was chosen as an environmentally relevant concentration (Cao et al., 2012; Dang and Wang, 2009; Environmental Protection Agency, 2001). The internal cadmium concentration measured in *H. diversicolor* used as a contaminated diet source to juvenile soles during the uptake phase was 0.2 ± 0.02 µg Cd $g^{-1}$ of organism, while no significant amount of cadmium (0.008 µg Cd $g^{-1}$) was detected to the non-contaminated *H. diversicolor* provided as clean diet during the depuration phase.

### 5.3.4. Basic set-up of the recirculated modular system (RMS)

The recirculated modular systems were assembled using materials and equipment readily available in local or online stores, thus facilitating its replication across the world (see **Figure 5.1**). A total of 4 RMS were used and each system module was composed of one high-density polyethylene food-grade (HDPE) tank (0.800 m long, 0.450 m wide, and 0.150 m high) with a maximum functional volume of approximately 36 L, connected to a HDPE filtration tank (0.6 m long, 0.4 m wide, and 0.3 m high), operating with a maximum functional water volume of approximately 36 L. The tank was divided in 8 compartments (0.200 m long, and 0.225 m wide) build with perforated plates of HDPE. The RMS operated in recirculated system with ASW. Filtered water in the filtration tank was pumped through a polyvinyl chloride (PVC) inlet pipe system by a submerged water pump (Eheim Compact 1000, Germany) with a regulated total flow of approximately 800 L $h^{-1}$. Each compartment, stocked with one *S. senegalensis*, was supplied in parallel with filtered ASW through one individual inlet pipe, derived from the main inlet pipe manifold system. Mechanical filtration was ensured by a FSI - XOI 50 µm filter bag (Tropical Marine Center, UK) connected to the outlet pipes, from the main tank to the filtration tank. Biological filtration was ensured by submerged bioballs (approximately 10 L). Experiments were

performed in a temperature-controlled room. No sediment was provided in order to avoid the loss of metals through adsorption and to reduce stress (soles were reared in the commercial hatchery in white sediment-free shallow tanks). Temperature was maintained at 20 ± 1 ºC and salinity at 35 ± 1. Water chemistry was maintained under optimal reference levels for *S. senegalensis* aquaculture during the experiment ($NH_3/NH_4^+$<1 mg $L^{-1}$, $NO_2^-$=0 mg $L^{-1}$, $NO^{3-}$<10 mg $L^{-1}$, $PO_4^{3-}$=0 mg $L^{-1}$, $Ca^{2+}$=420-480 mg $L^{-1}$, pH=7.9).

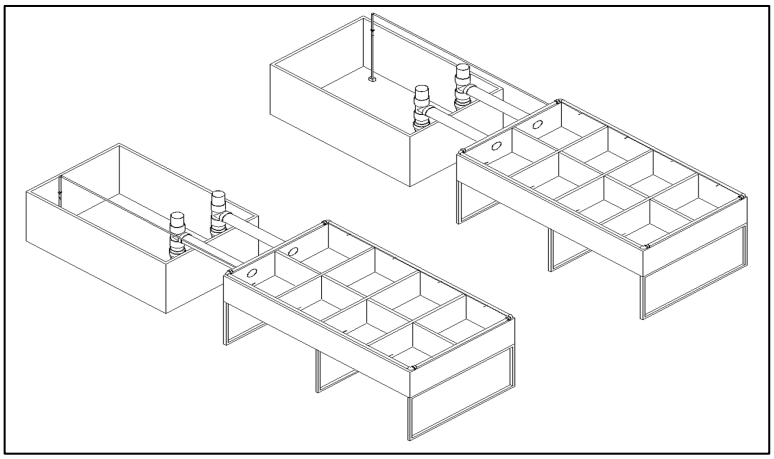

**Figure 5.1** Recirculated Modular System (RMS). Each module is divided in 8 compartments. Each compartment is supplied in parallel with filtered ASW through one individual inlet pipe, derived from the main inlet pipe manifold system.

### 5.3.5. Experimental Design – Dietary and Water Exposure

Acclimatization and experimental period followed the OECD guideline 305 (2012) for testing of bioaccumulation in fish, adapted for the marine species used in the present study. After the acclimatization period, a two-phase experiment was conducted, consisting on an uptake phase where 32 juvenile *S. senegalensis* were exposed to cadmium-contaminated ASW and fed with cadmium-contaminated polychaetes, followed by a depuration phase where fish were transferred to clean ASW and provided with non-contaminated polychaetes. Each uptake and depuration phase lasted for 14 days (for a total experimental period of 28 days). Juvenile soles were fed daily with 1% of their wet body weight, in order to maintain a constant body lipid content (OECD, 2012). Polychaetes provided during the uptake phase were previously exposed for 48 h to the same cadmium concentration as the soles used in the present experiment. Non-contaminated polychaetes were kept in clean ASW. The bottom of the RMS was cleaned

daily throughout the experiment to remove any uneaten food and fecal pellets. The required amount of fresh ASW contaminated with cadmium (uptake phase) or not contaminated (depuration phase) was added to compensate for the volume removed (~7 L) during daily cleaning procedures. Additionally, 90% of the ASW medium was renewed every 4 days to guarantee an ideal water quality within the test system. The uptake phase comprised six sampling times (at days 0, 1, 3, 7, 10, and 14) whereas the depuration phase included four sampling times (at days 17, 21, 24, and 28). Each sampling point consisted of 3 replicates and for every replicate one juvenile sole was sampled. Juvenile soles were anesthetized by placing them in ice and euthanized by decapitation prior to dissection. Two major tissue fractions were sampled: edible (muscle) and non-edible (intestine, gills and liver). All sampled tissues were rinsed with ultrapure water, snap frozen in liquid nitrogen and stored at -80 ºC until toxicokinetics analysis.

### 5.3.6. Chemical Analysis

Chemical analysis of the water was performed using inductively coupled plasma-mass spectrometry (ICP-MS) for cadmium. Samples from the stock solution and from the concentration tested were acidified after spiking and sent to the Laboratory of Chemical Analysis (LCA), University of Aveiro, to assess contamination accuracy.

All tissues were rinsed with ultrapure water to remove excess of medium, freeze-dried for 48 h to a constant weight, weighed and then digested using a mixture of acids, $HNO_3$ and $HClO_4$ (v/v, Baker Ultrex II Ultra Pure), at a ratio of 7:1 using 4 heating cycles (Method described in Chapter 3) in order to destroy all organic material and enable metal measurements being present in the final solution as ions. The residues were taken up with 1 mL of 0.1 M $HNO_3$ (Baker Ultrex II Ultra Pure) and cadmium content was measured using Graphite Furnace Atomic Absorption Spectrophotometry (Perkin-Elmer PinAAcle 900Z). Values presented here are calculated and corrected according to the dry weight of the organisms. For every digestion cycle, three replicates of blanks and three replicates of certified reference material (CRM) (DOLT-5, Dogfish liver CRM for trace metals and other constituents) were used to control for the accuracy of the method (cadmium concentration, 14.5 ± 0.6 mg $kg^{-1}$). Detection limit was 0.186 µg Cd $L^{-1}$ (n = 20). Recovery of cadmium from the certified reference material was 108%.

### 5.3.7. Toxicokinetic Models

The uptake and depuration kinetics of cadmium in the soles tissues exposed to both contaminated water and diet were described using first – order one – compartment model. The model assumed that the background concentration in each of the soles tissues is a fixed value $C_o$ at time zero and does not take part in the depuration. **Equation 5.1** represents the uptake phase while **Equation 5.2** represents the depuration phase.

For the uptake phase the model used reads:

$Q(t) = Co + \frac{u_f}{k_2} * (1 - e^{(-k_2 * t)})$ (**Equation 5.1**)

For the depuration phase the model used reads:

$Q(t) = Co + \frac{u_f}{k_2} * (e^{(-k_2 * (t - t_0))} - e^{(-k_2 * t)})$ (**Equation 5.2**)

where, Q(t) is the internal body burden in the tissue in μg of Cd $g^{-1}$ of dried body weight of each tissue in sampling time t,

$C_o$ is the initial (background) concentration in the sole's tissue in μg of Cd $g^{-1}$ of dried body weight of each tissue at time 0,

$u_f$ is the total uptake flux ($u_m$, water uptake flux and $u_d$, diet uptake flux) into the sole's tissue in μg of $Cd^{2+}$ $g^{-1}$ $day^{-1}$ (Vijver et al., 2006),

$k_2$ is the depuration rate parameter per day,

$t_0$ is the time at which organisms where transferred to freshly prepared uncontaminated water and diet in days, and

t is the sampling time in days.

Both equations used for describing the cadmium uptake and depuration patterns were fitted simultaneously, as suggested by the Guideline 305 (OECD, 2012).

### 5.3.8. Statistical Analysis

The kinetic parameters used by the model were calculated using non-linear regression analysis by fitting simultaneously the uptake and depuration equations to the data in the SPSS Statistical Package version 20.

The time (expressed in days) each tissue required to eliminate half the amount of cadmium ($DT_{50}$), was calculated as:

$DT_{50} = \frac{\ln(2)}{k_2}$ (**Equation 5.3**),

The concentration of cadmium at steady state ($C_{org}^{ss}$) in μg $g^{-1}$ in the organism was derived from the model **Equation 5.1** by substituting t = ∞.

### 5.3.9. Health Risk Assessment

The estimation of THQ for potential non-carcinogenic effects of cadmium in human health followed the methodology provided by US EPA Region III Risk-Based Concentration Table (United States Environmental Protection Agency (US EPA), 2010), with THQ being calculated as follows:

$THC = \frac{EF*ED*FIR*C_m}{RfD*BW*AT}$ (**Equation 5.4**)

where, EF is the exposure frequency in days year$^{-1}$ (365 days year$^{-1}$),

ED is the exposure duration in years,

FIR is the food ingestion rate in kg day$^{-1}$ person$^{-1}$(0.0334 for the world (FAO Fisheries, 2009) and 0.1559 for Portugal (EC, 2008)),

$C_m$ is the metal concentration in fish at steady state in mg kg$^{-1}$,

RfD is the reference dose in mg kg$^{-1}$ day$^{-1}$ (0.001 for cadmium (United States Environmental Protection Agency (US EPA), 2010)),

BW is the body weight in kg, and

AT is the average exposure time for non-carcinogens (365 days year$^{-1}$ x ED).

In the current study we used the same exposure duration values and body weight as Vieira et al (2011) according to US EPA (2008) for nine different age groups: 1 year and 14 kg (ages 1 to 3 years), 4 years and 21 kg (ages 4 to 6), 7 years and 32 kg (children 7 to 10 years), 11 years and 51 kg (adolescents 11 to 14 years), 15 years and 67 kg (adolescents 15 to 19 years), 20 years and 72 kg (adults 20 to 24 years), 25 years and 77 kg (adults 25 to 54 years), 55 years and 77 kg (adults 55 to 64 years) and 66 years and 72 kg (seniors >65 years).

The Estimated Weekly Intake (EWI) of cadmium was calculated using the following equation:

$EWI = \frac{Cm*WIR}{BW}$ (**Equation 5.5**)

where, $C_m$ is the metal concentration in fish at steady state in mg kg$^{-1}$,

WIR is the weekly food ingestion rate in kg week$^{-1}$ person$^{-1}$(0.2338 for the world (FAO Fisheries, 2009) and 1.0913 for Portugal (EC, 2008)), and

BW is the body weight in kg.

## 5.4. Results

### 5.4.1. Chemical Analysis

Results from cadmium chemical analysis in the stock and test solution showed a variation of 2 and 6%, respectively from the nominal ones, thus confirming the accuracy of the spiking technique. The Visual MINTEQ equilibrium model estimated the percentage of free ionic cadmium to be 4.5%, free ion concentration at 0.31 μg $Cd^{2+}$ $L^{-1}$ and free ionic activity at 0.09 μg $Cd^{2+}$ $L^{-1}$.

No fish mortality was recorded during the 14 days of acclimatization, neither during the 28 days of the whole bioassay.

### 5.4.2. Toxicokinetics of cadmium in soles

After 14 days of cadmium uptake none of the tissue's internal concentration reached a steady state phase and soles exposed to cadmium through water and diet presented differences in the accumulation patterns of each tissue. An increase in cadmium internal concentration was recorded for all tissues over time, with the lowest one recorded being for the muscle, and followed by the gills, liver and intestine.

**Table 5.1** Uptake and depuration kinetics parameters of cadmium in the muscle, gills, liver and intestine of juvenile *Solea senegalensis* exposed to both cadmium-contaminated water (ASW) and diet (polychaetes *H. diversicolor*). $C_o$ is the initial (background) concentration in the sole's tissues; $u_f$ is the total uptake flux ($u_m$, water uptake flux and $u_d$, diet uptake flux) into the sole's tissues; $k_2$ is the depuration rate; $DT_{50}$ is the time each tissue required to eliminate half the amount of cadmium; and $C_{org}^{ss}$ is the concentration of cadmium at steady state (95% CI in brackets).

| Fish Tissue | $C_o$ (μg Cd $L^{-1}$) | $u_f$ (μg $Cd^{2+}$ $g^{-1}$ $day^{-1}$) | $k_2$ ($day^{-1}$) | $DT_{50}$ (days) | $C_{org}^{ss}$ (μg Cd $L^{-1}$) |
|---|---|---|---|---|---|
| **Muscle** | 0.00 | 0.002 (0.001-0.003) | 0.11 (0.03-0.20) | 6.1 | 0.02 |
| **Gills** | 0.08 | 0.03 (0.02-0.04) | 0.02 (0-0.04) | 38.5 | 1.60 |
| **Liver** | 0.50 | 0.01 (0.002-0.02) | -0.05 (-) | - | - |
| **Intestine** | 0.21 | 0.36 (0.13-0.59) | 0.14 (0.03-0.24) | 5.1 | 2.86 |

Muscle: Cadmium concentration in the muscle of juvenile soles was 0.003 µg Cd $g^{-1}$ (not possible to calculate STDV/SE, n=1, as all other samples from day 14 were lost during digestions) at the end of the uptake phase (**Figure 5.2**). The kinetic parameters for cadmium ions activity obtained by a first-order one-compartment model are presented in **Table 5.1**. A slower uptake flux was estimated when compared to the rest of sampled tissues, while the depuration rate showed a faster elimination of cadmium from the muscle. The toxicokinetics model estimated the steady state concentration for this tissue to be 0.02 µg Cd $g^{-1}$.

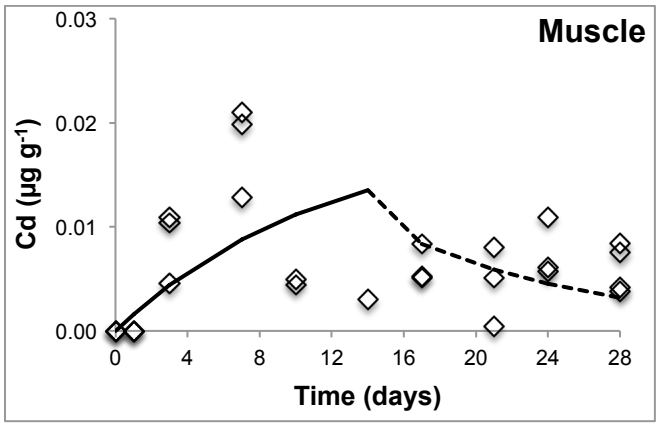


**Figure 5.2** Uptake and elimination kinetics of cadmium in the muscle of juvenile *Solea senegalensis* exposed to cadmium-contaminated water (ASW) and diet (polychaete *Hediste diversicolor*). Diamonds represent measured cadmium concentrations in muscle, the continuous line represents the predicted uptake in time and the dotted line represents the predicted depuration, using toxicokinetic models.

Gills: Cadmium concentration in gills increased with time during the uptake phase, with cadmium mean concentration being 0.34 µg Cd $g^{-1}$ (STDV=0.06, SE=0.03) after 14 days of exposure (**Figure 5.3**). The kinetic parameters for cadmium ions activity obtained by a first-order one-compartment model are presented in **Table 5.1**. Gills showed the third highest uptake flux rate and the second lowest depuration rate when compared to the rest of the tissues sampled in the present study. The toxicokinetics model estimated the steady state concentration for this tissue to be 1.60 µg Cd $g^{-1}$.

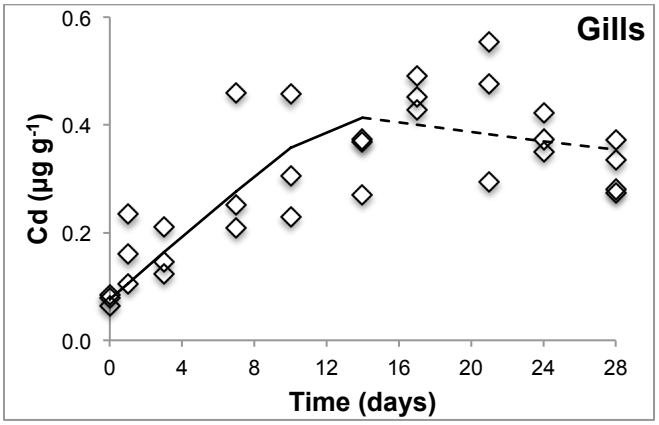


**Figure 5.3** Uptake and elimination kinetics of cadmium in the gills of juvenile *Solea senegalensis* exposed to cadmium-contaminated water (ASW) and diet (polychaete *Hediste diversicolor*). Diamonds represent measured cadmium concentrations in gills, the continuous line represents he predicted uptake in time and the dotted line represents the predicted depuration, using toxicokinetic models.

Liver: During the 14 days of uptake, the internal liver concentration did not reach a steady state phase, while it increased with time during the uptake phase. Mean cadmium concentration in the liver of juvenile soles was 0.76 µg Cd $g^{-1}$ (STDV=0.17, SE =0.1) at the end of the uptake (**Figure 5.4**). The kinetic parameters for cadmium ions activity obtained by a first-order one-compartment model are presented in **Table 5.1**. A negative depuration rate constant cannot be explained biologically and therefore was considered zero for further calculations, while the uptake flux of cadmium to the liver was the second highest. No half-life for cadmium was calculated for this specific tissue due to the negative/zero depuration rate; moreover, the toxicokinetics model was not able to estimate a steady state concentration, as the internal concentration would keep increasing indefinitely.

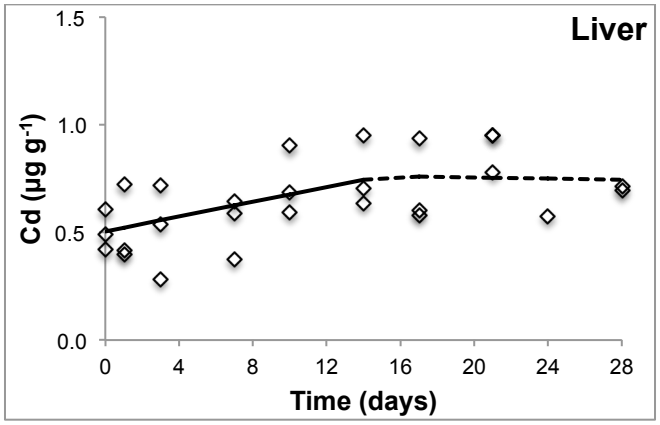


**Figure 5.4** Uptake and elimination kinetics of cadmium in the liver of juvenile *Solea senegalensis* exposed to cadmium-contaminated water (ASW) and diet (polychaete *Hediste diversicolor*). Diamonds represent measured cadmium concentrations in liver, the continuous line represents the predicted uptake in time and the dotted line represents the predicted depuration, using toxicokinetic models.

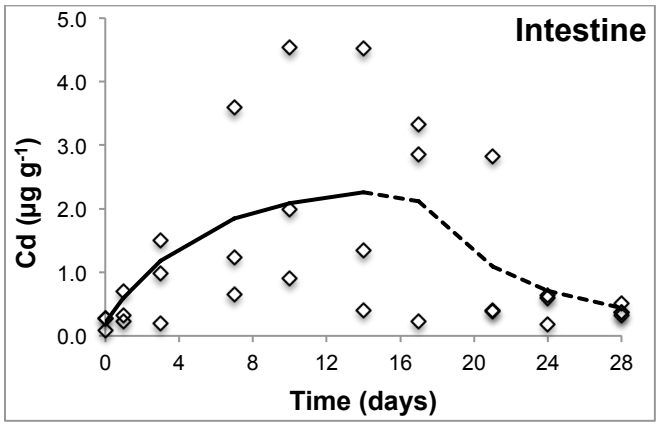


**Figure 5.5** Uptake and elimination kinetics of cadmium in the intestine of juvenile *Solea senegalensis* exposed to cadmium-contaminated water (ASW) and diet (polychaete *Hediste diversicolor*). Diamonds represent measured cadmium concentrations in the intestine, the continuous line represents the predicted uptake in time and the dotted line represents the predicted depuration, using toxicokinetic models.

Intestine: After 14 days of uptake, cadmium concentration in the tissue increased with time. Mean internal concentration in the intestine of juvenile soles was 2.1 µg Cd $g^{-1}$ (STDV=2.2, SE =1.2). No steady state on the intestine concentration was reached during the uptake phase (**Figure 5.5**). Values of the kinetic parameters for cadmium ions activity obtained by a first-order one-compartment model are presented in **Table 5.1**. Fish

intestine showed the highest cadmium uptake flux and depuration rate when compared to the rest of the tissues analyzed in the present study. A steady state concentration of 2.86 μg Cd $g^{-1}$ was estimated by the toxicokinetics model.

### 5.4.3. Health Risk Assessment

The concentrations used to estimate the THQ values in the present study were lower than the maximum acceptable limit set by the EU Commission Regulation (EC) No 1881/2006 (EC, 2006) at 0.05 mg/kg wet weight. THQ values for cadmium are lower than one for juvenile *S. senegalensis* used in the present study. EWI values, when considering world consumption, ranged from as low as 0.009 to 0.334, while those for the Portuguese population reached as high as 1.559. THQ and EWI values are summarized in **Table 5.2**.

**Table 5.2** Risk assessment for non-carcinogenic effects in humans by cadmium measured in the muscle of juvenile *Solea senegalensis* using Estimated Weekly Intakes (EWI) (μg $kg^{-1}$ body weight) and Target Hazard Quotients (THQ) for different age groups. Provisional Tolerable Weekly Intake (PTWI) of cadmium is set at 2.5 μg $kg^{-1}$ body weight by EFSA (2012).

| Age Group | EWI (1) | THQ (1) | EWI (2) | THQ (2) | EWI (3) | THQ (3) | EWI (4) | THQ (4) |
|---|---|---|---|---|---|---|---|---|
| **Children 1-3 yrs** | 0.050 | 0.007 | 0.234 | 0.033 | 0.334 | 0.048 | 1.559 | 0.223 |
| **Children 4-6 yrs** | 0.033 | 0.005 | 0.156 | 0.022 | 0.223 | 0.032 | 1.039 | 0.148 |
| **Children 7-10 yrs** | 0.022 | 0.003 | 0.102 | 0.015 | 0.146 | 0.021 | 0.682 | 0.097 |
| **Adolescents 11-14 yrs** | 0.014 | 0.002 | 0.064 | 0.009 | 0.092 | 0.013 | 0.428 | 0.061 |
| **Adolescents 15-19 yrs** | 0.010 | 0.001 | 0.049 | 0.007 | 0.070 | 0.010 | 0.326 | 0.047 |
| **Adults 20-24 yrs** | 0.010 | 0.001 | 0.045 | 0.006 | 0.065 | 0.009 | 0.303 | 0.043 |
| **Adults 25-54 yrs** | 0.009 | 0.001 | 0.043 | 0.006 | 0.061 | 0.009 | 0.283 | 0.040 |
| **Adults 54-64 yrs** | 0.009 | 0.001 | 0.043 | 0.006 | 0.061 | 0.009 | 0.283 | 0.040 |
| **Seniors > 65 yrs** | 0.010 | 0.001 | 0.045 | 0.006 | 0.065 | 0.009 | 0.303 | 0.043 |

(1) THQ/EWI values according to World fish consumption using cadmium concentration in muscle after 14 days

(2) THQ/EWI values according to Portugal's fish consumption using cadmium concentration in muscle after 14 days

(3) THQ/EWI values according to World's fish consumption using cadmium concentration in muscle at steady state

(4) THQ/EWI values according to Portugal's fish consumption using cadmium concentration in muscle at steady state

## 5.5. Discussion

While an increasing attention has been devoted to the patterns of metal accumulation in marine organisms, few studies have considered the effects of multiple exposure routes. When it comes to marine fish, the gap of knowledge on this research field is even more concerning, as to the authors' best knowledge no studies exist addressing the effects of a simultaneous exposure to both cadmium-contaminated water and diet. International guidelines, such as OECD Guideline 305 (OECD, 2012), only refer bioaccumulation of metals in fish either through water or diet, which makes the estimation of bioaccumulation and pathway contribution in case of a two route exposure, a challenging task.

Contaminants in fish may constitute a threat to the organism itself, as well as to tertiary consumers, such as humans. Considering that the relative contribution of different uptake routes remain poorly understood to marine fish, and are likely to influence metal distribution over their body tissues, an integrated assessment is critical, particularly in species used for human consumption (directly as food or indirectly as feed for other animals destined for human consumption). In the present study, an attempt to estimate and predict the accumulation patterns of cadmium in the marine flatfish, *S. senegalensis*, under simultaneous exposure to cadmium contaminated water and diet was made.

The exposure of soles to cadmium during 14 days did not show to be enough for any of the tissues' internal concentration to reach a steady state, nor did the 14 days of depuration seem to be enough for cadmium to be completely eliminated. These findings are in agreement with previous studies that showed the increased capacity that marine fish have to bioaccumulate and store cadmium, as well as and their limited ability to eliminate it from their body (Creighton and Twining, 2010; Schultz et al., 1996; Souid et al., 2013; Zhang and Wang, 2007). The bioaccumulation patterns and kinetic parameters recorded for cadmium exhibited considerable differences between the different tissues sampled in the present study. Such variability can be attributed to the fact of cadmium displaying a specific affinity to different tissues (Jezierska and Witeska, 2006; Schultz et al., 1996). Cadmium accumulation in juvenile *S. senegalensis* tissues followed a similar pattern of distribution to those previously described for other fish (Cao et al., 2012; Kalman et al., 2010; Kim et al., 2006), overall demonstrating higher concentrations of the metal to be present in the intestine followed by the liver, the gills and lastly the muscle. The intestine has been considered a relevant target for cadmium, for both waterborne and dietary routes of exposure, presenting therefore a significant accumulated cadmium burden (Jezierska and Witeska, 2006). Previous studies, where only a single exposure

route was considered, have shown that apart from the intestine, dietary cadmium tends to be mostly accumulated in the kidney and liver, whereas waterborne cadmium is more prone to be accumulated in the gills (Jezierska and Witeska, 2006; Kim et al., 2006; 2004). Several authors have reported the same patterns of cadmium accumulation in other marine fish, either from field or from laboratory based studies, when exposed to dietary or waterborne contamination (Cao et al., 2012; Dang and Wang, 2009; Long and Wang, 2005; Marcovecchio et al., 1988). Independently of the routes of exposure, cadmium tends to accumulate mainly in the liver and kidney, as well as the intestine and the gills (Kim et al., 2006).

Muscle: The edible fraction of Senegalese soles showed the slowest uptake rates, as well as the lowest accumulated cadmium concentration, among all sampled tissues. The concentration of cadmium measured in the muscle of juvenile *S. senegalensis* after exposed to an environmentally relevant concentration was lower to the maximum acceptable limit set by the EU Commission Regulation (EC) No 1881/2006 (EC, 2006) at 0.05 mg/kg wet weight. Muscle is one of the tissues, where the least amount of cadmium is usually accumulated (Long and Wang, 2005), most likely due to the comparatively lower blood circulation that occurs in this specific tissue. Vieira et al (2011) reported values of cadmium in the edible fraction of three commercially available fish (*Sardina pilchardus*, *Scober japonicus* and *Trachurus trachurus*) caught in Portuguese waters and purchased from local markets, ranging from 0.0056 to 0.0084 mg $kg^{-1}$, wet weight. Cao et al (2012) reported cadmium accumulation in muscle tissues to be the lowest when compared to gills, liver and kidney and ranging from 0.25 to 0.66 μg $g^{-1}$ upon Japanese flounders, *Paralichthys olivaceus*, exposure to waterborne cadmium concentrations ranging from 2 to 8 mg $g^{-1}$ for 28 days. Cadmium accumulation in the muscle did not seem to significantly change with time after exposure of *P. olivaceous* to low dosed cadmium (up to 50 μg $L^{-1}$). To date, available results suggest that muscle may not be an appropriate bioindicator tissue for environmental cadmium concentrations as the accumulated cadmium in the muscle is not always proportional to the environmental cadmium contamination.

Gills: The gills play a key role in fish physiology, as they are responsible for ion, acid-base regulation and gas exchange, as well as for the excretion and elimination of nitrogenous waste products (Di Toro et al., 2001; Hogstrand and Wood, 1998; Paquin et al., 2002a; 2002b). Even though, it has been proven that high concentrations of cadmium are needed to disrupt gas exchange and ionoregulation and cause severe gill pathology, low environmentally relevant concentrations may negatively affect ions regulatory process and subsequently induce toxicity to fish (Hogstrand and Wood, 1998). $Cd^{2+}$ tends to bind to

the gills at specific sites (biotic ligand) inhibiting $Ca^{2+}$ uptake and metabolism and ultimately disrupt the integrity of the epithelium (Paquin et al., 2002a). Gills are in direct contact with waterborne cadmium (Cao et al., 2012) and possibly be transferred and redistributed through the circulatory system to the rest of the fish body and vice versa (Pratap and Wendelaar-Bonga, 1993). Slow depuration of cadmium from gills could indicate such pattern, as assimilated cadmium, either waterborne or dietary, by the intestine can enter the body circulation through blood and reach the gills causing damage to chloride cells (Kim et al., 2006; Pratap and Wendelaar-Bonga, 1993). Several authors have reported elevated values of cadmium in the branchial epithelium of fish exposed to waterborne or dietary cadmium due to the induction of metallotheionein-like proteins synthesis (MTLPs) as a detoxification mechanism, and those proteins are usually found stored in the chloride cells of the gill (Dang and Wang, 2009; Dang et al., 2001; Hogstrand and Wood, 1998; Kim et al., 2006).

Liver: High cadmium concentration in the liver can be attributed to the fact that this particular organ significantly contributes to the detoxification and storage of cadmium in fish (Cao et al., 2012; Kim et al., 2006; 2004). Along with the kidney, liver is known to be related with the production and storage of MTLPs, as well as the existence of gluthathione (GSH) in the liver cytosol, responsible for binding to metals as a detoxification mechanism through metal sequestration (Jebali et al., 2013; Kalman et al., 2010; Schultz et al., 1996; Williams et al., 2006). Jebali et al (2013) support the theory that MTs are tissue specific and showed that the highest content was in the kidney followed by the liver and the gills. No depuration was observed in the present study from this particular tissue, whereas an increase in the internal concentration was observed even during the depuration phase. It is likely that during depuration MTs and GSH are detoxifying cadmium by binding it and subsequently storing it in the liver, thus rendering it inert (Kim et al., 2004).. The inability of this particular organ to eliminate cadmium can lead to hepatotoxicity; this is likely due to the depletion of MTs and GSH promoted by the “spill-over” phenomenon (Kalman et al., 2010), as cadmium is considered a hepatotoxicant (Williams et al., 2006). Liver has been previously proposed as a suitable bioindicator of water contamination as it bioaccumulates cadmium and reflects the concentrations found in the surrounding environment (Jezierska and Witeska, 2006).

Intestine: The highest cadmium concentration after waterborne and dietary exposure was found in the intestine, approximately a 3-fold increase compared to the liver and a 6-fold increase when compared to the gills. Such outcome was somehow expected due to the direct contact/passage of the diet through the intestine, after initial digestion in the

stomach, and the high assimilation efficiency of nutrients and metals displayed by this particular organ (Dinis et al., 2000). Exposure of Atlantic cod, *Gadus morhua*, to cadmium indicated a 3-fold difference in the concentration measured in the intestinal mucosa when compared to the gills, suggesting that the intestine might be the primary uptake site for several waterborne metals, including cadmium (Hogstrand and Wood, 1998). Marine fish have a higher drinking rate (aprox. 0.5% of their body weight per hour) when compared to their freshwater counterparts, due to the hyperosmotic environment they inhabit. This environmental driver may be responsible to the higher proportion of total cadmium that accumulates in the intestine as result from waterborne exposure (Cao et al., 2012; Kim et al., 2004; Long and Wang, 2005). Kuroshima (1992) observed that preferential cadmium accumulation in the killifish *Fundulus heteroclitus* switched from the gills to the intestine with increasing salinities, thus suggesting a higher metal uptake rate through the ingestion of seawater to maintain osmotic balance. However, dietary uptake also plays a significant role in the cadmium load present in the fish intestine. Dang and Wang (2009) showed significant differences in cadmium bioaccumulation in the digestive tract of marine grunts, *Terapon jarbua*, after 14 days when exposed to increasing dietary cadmium compared to waterborne contamination. However, the same authors also reported that in long-term exposures (4 weeks) cadmium bioaccumulation in the intestine was mainly due to waterborne cadmium rather than dietary. Such result could be due to the distinct routes of cadmium uptake and/or depuration by the intestine, as it may depend on the form cadmium is stored in the prey (e.g. TAM-Trophically Available Metals) and/or the digestive and assimilative efficiency of the fish. Kalman et al. (2010) suggested that the intestinal walls have the capacity to store cadmium and may latter be involved in the elimination and/or redistribution of the metal to the rest of the tissues. Dang and Wang (2009) showed that MT production in the intestine induced by either waterborne or dietary cadmium after 14 days of exposure, was considerably higher when compared to the gills or the liver.

The biological half-life of cadmium in the tissues surveyed in the present study, with the exception of the liver, varied from 6 to 39 days, while other studies have reported estimates ranging from 24 to 200 days (Schultz et al., 1996 and references therein). These differences can be attributed to phylogenetic issues (different species), differences in the age and size of monitored specimens (e.g., juvenile *vs.* adults), routes for cadmium exposure (e.g., waterborne *vs.* dietary), as well as different abiotic conditions (e.g., temperature, pH, salinity, zinc concentration, presence of organic matter, amongst others).

### 5.5.1. Health Risk Assessment

The analysis of non-carcinogenic risks has showed THQ values <1 to all age groups exposed in the concentrations found in the current study. Such results indicated that an exposed population could be considered safe if consuming fish with the amount of cadmium measured in the current study. Vieira et al. (2011) suggested a more intensive selection and compilation of information and data, concerning real ingestion rates. Children's intake rate is often higher comparable to their body weight than that of an adult and exposure frequency for each species consumed and age groups can be refined, so these estimates can be better improved.

The European Food Safety Agency (EFSA) (2012) has recommended a Provisional Tolerable Weekly Intake (PTWI) of cadmium of 2.5 μg $kg^{-1}$ body weight. All EWIs estimated here were within the PTWI levels proposed by EFSA (2012). The Portuguese population is estimated to be ingesting weekly higher levels of cadmium compared to the mean world population. Such result would be expected due to a higher consumption of seafood compared to the mean world value (FAO Fisheries, 2009; EC, 2008). Values of ca. half the proposed maximum limits were estimated for the Portuguese population in the youngest age groups (children 1-3 yrs and children 4-6 yrs) when compared to older ones. Decreasing EWI with increasing age would result from the dilution that the metal undergoes due to increasing body weight when compared to younger age groups.

Comparisons between the maximum acceptable values for cadmium in the muscle, THQ and EWI shows that the cadmium measured in the muscle of juvenile soles from the present study is within acceptable limits and can be considered safe for human consumption.

## 5.6. Conclusions

The bioaccumulation profile of cadmium in *S. senegalensis* differs with the tissue being analyzed. Upon cadmium exposure from contaminated diet and water, the highest concentration for this metal was found in the intestine and the liver, while the muscle showed a very low concentration. The liver showed no cadmium depuration while depuration was observed for other tissues, which was probably due to its detoxifying and storage role in fish. Gills showed a high binding capacity of cadmium also during the depuration rate, suggesting an internalization of cadmium in cells and a probable redistribution through the plasma. Even though soles from cadmium-contaminated

environments could be safe for human consumption due to low cadmium levels in the muscle, as THQ values reported are below one, they may comprise a potential risk to higher predators, such as bigger fish, birds or mammals. This study has provided indications that bioaccumulation through a two way exposure scenario is an important parameter to take into consideration when assessing non-essential metals like cadmium. Further studies using higher exposure concentrations, field populations and assessing subcellular compartmentalization to prey and individual tissues would be advised in order to clearly understand the uptake routes and storage preferences of cadmium.

## 5.7. References

FAO Fisheries, 2009. The state of world fisheries and aquaculture 2008.

Barhoumi, S., Messaoudi, I., Deli, T., Saïd, K., Kerkeni, A., 2009. Cadmium bioaccumulation in three benthic fish species, *Salaria basilisca*, *Zosterisessor ophiocephalus* and *Solea vulgaris* collected from the Gulf of Gabes in Tunisia. Journal of Environmental Sciences 21, 980–984. doi:10.1016/S1001-0742(08)62371-2

Bjerregaard, P., Depledge, M.H., 1994. Cadmium accumulation in *Littorina littorea*, *Mytilus edulis* and *Carcinus maenas*: the influence of salinity and calcium ion concentrations. Mar. Biol. 119, 385–395. doi:10.1007/BF00347535

Burgos, M.G., Rainbow, P.S., 2001. Availability of cadmium and zinc from sewage sludge to the flounder, *Platichthys flesus*, via a marine food chain. Mar Environ Res 51, 417–439. doi:10.1016/S0141-1136(00)00249-X

Cabral, H., Costa, M.J., 1999. Differential use of nursery areas within the Tagus estuary by sympatric soles, *Solea solea* and *Solea senegalensis*. Environmental Biology of Fishes 56, 389–397. doi:10.1023/A:1007571523120

Cao, L., Huang, W., Shan, X., Ye, Z., Dou, S., 2012. Tissue-specific accumulation of cadmium and its effects on antioxidative responses in Japanese flounder juveniles. Environmental Toxicology and Pharmacology 33, 16–25. doi:10.1016/j.etap.2011.10.003

Commision, E.C., 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuff [WWW Document].

Costa, P.M., Lobo, J., Caeiro, S., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., DelValls, T.Á., Costa, M.H., 2008. Genotoxic damage in *Solea senegalensis* exposed to sediments from the Sado Estuary (Portugal): effects of metallic and organic contaminants. Mutat. Res. 654, 29–37. doi:10.1016/j.mrgentox.2008.04.007

Creighton, N., Twining, J., 2010. Bioaccumulation from food and water of cadmium, selenium and zinc in an estuarine fish, *Ambassis jacksoniensis*. Marine Poll Bull 60, 1815–1821. doi:10.1016/j.marpolbul.2010.05.025

Dang, F., Wang, W.-X., 2009. Assessment of tissue-specific accumulation and effects of cadmium in a marine fish fed contaminated commercially produced diet. Aquat. Toxicol. 95, 248–255. doi:10.1016/j.aquatox.2009.09.013

Dang, Z.C., Berntssen, M., Lundebye, A.K., Flik, G., Wendelaar Bonga, S.E., Lock, R., 2001. Metallothionein and cortisol receptor expression in gills of Atlantic salmon, *Salmo salar*, exposed to dietary cadmium. Aquat Toxicol 53, 91–101.

doi:10.1016/S0166-445X(00)00168-5

Di Toro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ Toxicol Chem 20, 2383–2396. doi:10.1002/etc.5620201034/full

Dinis, M.T., Ribeiro, L., Conceição, L., Aragão, C., 2000. Larvae digestion and new weaning experiments in *Solea senegalensis*. *Cah. Options Méditerr* 47 (2000): 193-204.

Dinis, M.T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. Aquaculture 176, 27–38. doi:10.1016/S0044-8486(99)00047-2

EC, E.C., 2008. Facts and figures on the CFP Basic data on the Common Fisheries Policy – Edition 2008. Luxembourg.

Eichinger, M., Loizeau, V., Roupsard, F., Le Guellec, A.M., Bacher, C., 2010. Modelling growth and bioaccumulation of Polychlorinated biphenyls in common sole (*Solea solea*). Journal of Sea Research 64, 373–385. doi:10.1016/j.seares.2010.05.005

European Food Safety Authority (EFSA), 2012. Cadmium dietary exposure in the European population, EFSA Journal. Eur Food Saf Authority (doi:10.2903/j.efsa.2012.2551

Figueira, E., Cardoso, P., Freitas, R., 2012. *Ruditapes decussatus* and *Ruditapes philippinarum* exposed to cadmium: toxicological effects and bioaccumulation patterns. Comp Biochem Physiol C 156, 80–86. doi:10.1016/j.cbpc.2012.04.004

Fonseca, V.F., França, S., Serafim, A., Company, R., Lopes, B., Bebianno, M.J., Cabral, H.N., 2011. Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*. Aquat. Toxicol. 102, 216–227. doi:10.1016/j.aquatox.2011.01.018

Fonseca, V.F., Vasconcelos, R.P., Tanner, S.E., França, S., Serafim, A., Lopes, B., Company, R., Bebianno, M.J., Costa, M.J., Cabral, H.N., 2015. Habitat quality of estuarine nursery grounds: Integrating non-biological indicators and multilevel biological responses in *Solea senegalensis*. Ecological Indicators 58, 335–345. doi:10.1016/j.ecolind.2015.05.064

Galindo, M.D., Jurado, J.A., García, M., de Canales, M.L.G., Oliva, M., López, F., Granado, M.D., Espada, E., 2012. Trace metal accumulation in tissues of sole (*Solea senegalensis*) and the relationships with the abiotic environment. International Journal of Environmental Analytical Chemistry 92, 1072–1092. doi:10.1080/03067319.2010.548092

Geffard, A., Smith, B.D., Amiard-Triquet, C., Jeantet, A.Y., Rainbow, P.S., 2005. Kinetics of trace metal accumulation and excretion in the polychaete *Nereis diversicolor*. Mar. Biol. 147, 1291–1304. doi:10.1007/s00227-005-0044-z

Gobas, F.A.P.C., Morrison, H.A., 2000. Bioconcentration and Biomagnification in the Aquatic Environment, in: Press, C. (Ed.), Handbook of Property Estimation Methods for Chemicals. pp. 189–197.

Goetze, S., Matoo, O.B., Beniash, E., Saborowski, R., Sokolova, I.M., 2014. Interactive effects of $CO_2$ and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. Aquat Toxicol 149, 65–82. doi:10.1016/j.aquatox.2014.01.027

Hogstrand, C., Wood, C.M., 1998. Toward a better understanding of the bioavailability, physiology and toxicity of silver in fish: Implications for water quality criteria. Environ Toxicol Chem 17, 547–561.

Imsland, A.K., Foss, A., Conceição, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P., White, P., 2003. A review of the culture potential of *Solea solea* and *S. senegalensis*. Rev Fish Biol Fisheries 13, 379–408. doi:10.1007/s11160-004-1632-6

Jebali, J., Sabbagh, M., Banni, M., Kamel, N., Ben-Khedher, S., M'hamdi, N., Boussetta, H., 2013. Multiple biomarkers of pollution effects in *Solea solea* fish on the Tunisia coastline. Environ Sci Pollut Res 20, 3812–3821. doi:10.1007/s11356-012-1321-2

Jezierska, B., Witeska, M., 2006. The metal uptake and accumulation in fish living in polluted waters, in: Soil and Water Pollution Monitoring, Protection and Remediation, NATO Science Series. Springer Netherlands, Dordrecht, pp. 107–114. doi:10.1007/978-1-4020-4728-2_6

Jung, K., Zauke, G.P., 2008. Bioaccumulation of trace metals in the brown shrimp Crangon crangon (Linnaeus, 1758) from the German Wadden Sea. Aquat Toxicol 88, 243–249. doi:10.1016/j.aquatox.2008.05.007

Kalman, J., Riba, I., Angel DelValls, T., Blasco, J., 2010. Comparative toxicity of cadmium in the commercial fish species *Sparus aurata* and *Solea senegalensis*. Ecotoxicol Environ Safety 73, 306–311. doi:10.1016/j.ecoenv.2009.10.013

Keating, J., Delaney, M., Meehan-Meola, D., Warren, W., Alcivar, A., Alcivar-Warren, A., 2007. Histological findings, cadmium bioaccumulation, and isolation of expressed sequence tags (ESTs) in cadmium-exposed, specific pathogen-free shrimp, *Litopenaeus vannamei* postlarvae. Journal of Shellfish Research 26, 1225–1237. doi:10.2983/0730-8000(2007)26[1225:HFCBAI]2.0.CO;2

Kim, S.-G., Eom, K.-H., Kim, S.-S., Jin, H.-G., Kang, J.-C., 2006. Kinetics of Cd

accumulation and elimination in tissues of juvenile rockfish (*Sebastes schlegeli*) exposed to dietary Cd. Mar Environ Res 62, 14–14. doi:10.1016/j.marenvres.2006.05.001

Kim, S.-G., Jee, J.-H., Kang, J.-C., 2004. Cadmium accumulation and elimination in tissues of juvenile olive flounder, *Paralichthys olivaceus* after sub-chronic cadmium exposure. Environ Pollut 127, 117–123. doi:10.1016/S0269-7491(03)00254-9

Kuroshima, R., 1992. Cadmium accumulation in the mummichog, *Fundulus heteroclitus*, adapted to various salinities. Bull Environ Contam Toxicol 49, 680–685. doi:10.1007/BF00200780

Leborans, G.F., Novillo, A., 1996. Toxicity and bioaccumulation of cadmium in *Olisthodiscus luteus* (Raphidophyceae). Water Res. 30, 57–62. doi:10.1016/0043-1354(95)00084-X

Long, A., Wang, W.-X., 2005. Assimilation and bioconcentration of Ag and Cd by the marine black bream after waterborne and dietary metal exposure. Environ Toxicol Chem 24, 709–716.

Long, A., Wang, W.X., 2005. Metallothionein induction and bioaccumulation kinetics of Cd and Ag in the marine fish *Terapon jarbua* challenged with dietary or waterborne Ag and Cu. Mar. Ecol. Prog. Ser. 291, 215–226. doi:10.3354/meps291215

Marcovecchio, J.E., Moreno, V.J., Perez, A., 1988. The sole, *Paralichthys* sp., as an indicator species for heavy metal pollution in the Bahia Blanca estuary, Argentina. Sci Total Environ 75, 191–199. doi:10.1016/0048-9697(88)90032-0

Mathews, T., Fisher, N.S., 2009. Dominance of dietary intake of metals in marine elasmobranch and teleost fish. Sci Total Environ 407, 5156–5161. doi:10.1016/j.scitotenv.2009.06.003

Metian, M., Hédouin, L., Eltayeb, M.M., Lacoue-Labarthe, T., Teyssié, J.-L., Mugnier, C., Bustamante, P., Warnau, M., 2010. Metal and metalloid bioaccumulation in the Pacific blue shrimp *Litopenaeus stylirostris* (Stimpson) from New Caledonia: Laboratory and field studies. Marine Poll Bull 61, 576–584. doi:10.1016/j.marpolbul.2010.06.035

Munger, C., Hare, L., 1997. Relative Importance of Water and Food as Cadmium Sources to an Aquatic Insect (*Chaoborus punctipennis*): Implications for Predicting Cd Bioaccumulation in Nature. Environ. Sci. Technol. 31, 891–895. doi:10.1021/es9606440

OECD, 2012. OECD Guidelines for the Testing of Chemicals, Section 3: Degradation and Accumulation Test No. 305: Bioaccumulation in Fish: Aqueous and Dietary Exposure. OECD Publishing. doi:10.1787/9789264185296-en

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G.C., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., McGeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B., 2002a. The biotic ligand model: a historical overview. Comp Biochem Physiol C 133, 3–35.

Paquin, P.R., Zoltay, V., Winfield, R.P., Wu, K.B., Mathew, R., Santore, R.C., Di Toro, D.M., 2002b. Extension of the biotic ligand model of acute toxicity to a physiologically-based model of the survival time of rainbow trout (*Oncorhynchus mykiss*) exposed to silver. Comp Biochem Physiol C 133, 305–343.

Pratap, H.B., Wendelaar Bonga, S.E., 1993. Effect of ambient and dietary cadmium on pavement cells, chloride cells, and Na+/K+-Atpase activity in the gills of the freshwater teleost *Oreochromis mossambicus* at normal and high calcium levels in the ambient water. Aquat Toxicol 26, 133–149. doi:10.1016/0166-445X(93)90010-X

Schultz, I.R., Peters, E.L., Newman, M.C., 1996. Toxicokinetics and disposition of inorganic mercury and cadmium in channel catfish after intravascular administration. Toxicol. Appl. Pharmacol. 140, 39–50. doi:10.1006/taap.1996.0195

Singh, R., Gautam, N., Mishra, A., Gupta, R., 2011. Heavy metals and living systems: An overview. Indian J Pharmacol 43, 246–253. doi:10.4103/0253-7613.81505

Sisman-Aydin, G., Buyukisik, B., Oral, R., 2013. Bioaccumulation of cadmium in marine diatom: *Thalassiosira allenii* Takano. Turkish Journal of Fisheries and Aquatic Sciences 13, 861–867. doi:10.4194/1303-2712-v13_5_10

Souid, G., Souayed, N., Yaktiti, F., Maaroufi, K., 2013. Effect of acute cadmium exposure on metal accumulation and oxidative stress biomarkers of *Sparus aurata*. Ecotoxicol Environ Safety 89, 1–7. doi:10.1016/j.ecoenv.2012.12.015

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metal toxicity and the environment., in: Luch, A. (Ed.), Molecular, Clinical and Environmental Toxicology, Experientia Supplementum. Springer Basel, Basel, pp. 133–164. doi:10.1007/978-3-7643-8340-4_6

United States Environmental Protection Agency (US EPA), 2010. Risk-Based Concentration Table [WWW Document]. http://www.epa.gov/reghwmd/risk/human/index.htm.

United States Environmental Protection Agency (US EPA), 2008. Child-Specific Exposure Factors Handbook (Final Report) (No. EPA/600/R-06/096F). Washington, DC.

United States. Environmental Protection Agency. Office of Water, United States. Environmental Protection Agency. Office of Science and Technology. 2001 Update of

Ambient Water Quality Criteria for Cadmium.

Velez, C., Figueira, E., Soares, A., Freitas, R., 2015. Spatial distribution and bioaccumulation patterns in three clam populations from a low contaminated ecosystem. Estuarine, Coastal and Shelf Science 155, 114–125. doi:10.1016/j.ecss.2015.01.004

Vieira, C., Morais, S., Ramos, S., Delerue-Matos, C., Oliveira, M.B.P.P., 2011. Mercury, cadmium, lead and arsenic levels in three pelagic fish species from the Atlantic Ocean: Intra- and inter-specific variability and human health risks for consumption. Food Chem. Toxicol. 49, 923–932. doi:10.1016/j.fct.2010.12.016

Vijver, M.G., Vink, J.P.M., Jager, T., van Straalen, N.M., Wolterbeek, H.T., van Gestel, C.A.M., 2006. Kinetics of Zn and Cd accumulation in the isopod *Porcellio scaber* exposed to contaminated soil and/or food. Soil Biol Biochem 38, 1554–1563. doi:10.1016/j.soilbio.2005.11.006

Wang, M.-J., Wang, W.-X., 2009. Cadmium in three marine phytoplankton: Accumulation, subcellular fate and thiol induction. Aquat Toxicol 95, 99–107. doi:10.1016/j.aquatox.2009.08.006

Wang, M.-J., Wang, W.-X., 2008. Cadmium toxicity in a marine diatom as predicted by the cellular metal sensitive fraction. Environ. Sci. Technol. 42, 940–946. doi:10.1021/es0719273

Williams, T.D., Diab, A.M., George, S.G., Godfrey, R.E., Sabine, V., Conesa, A., Minchin, S.D., Watts, P.C., Chipman, J.K., 2006. Development of the GENIPOL European flounder (*Platichthys flesus*) microarray and determination of temporal transcriptional responses to cadmium at low dose. Environ. Sci. Technol. 40, 6479–6488. doi:10.1021/es061142h

Xu, Y., Wang, W.-X., Hsieh, D.P.H., 2001. Influences of metal concentration in phytoplankton and seawater on metal assimilation and elimination in marine copepods. Environ Toxicol Chem 20, 1067–1077. doi:10.1002/etc.5620200518

Zhang, L., Wang, W.-X., 2007. Gastrointestinal uptake of cadmium and zinc by a marine teleost *Acanthopagrus schlegeli*. Aquat Toxicol 85, 143–153. doi:10.1016/j.aquatox.2007.08.008

# Chapter 6
# General Discussion and Conclusions

## 6.1. Summary and Final Discussion

Assessing the potential toxicity of contaminants entering the marine environment, such as metals, is a challenging task. In order to employ reliable approaches and ensure ecological relevance, researchers should focus, whenever possible, in using a number of target species representing different trophic levels. One of the main shortcomings that ecotoxicologists encounter when assessing the toxicity of metals in the marine environment is the remarkable complexity of chemical processes that metals undergo under the influence of seawater (Wood et al., 2012). Despite the increasing number of scientific reports in marine ecotoxicology and the growing interest in the last decades on this topic, namely through the testing of different scenarios using metals and marine organisms, and the constant suggestions for a more elaborative and precise development of international guidelines and standards for the marine environment, aquatic ecotoxicology remains strongly biased towards its freshwater segment (Mhadhbi et al., 2010; Pérez et al., 2016; Wood et al., 2012). Hence, this thesis intended to assess the eco-, genotoxicity and bioaccumulation of cadmium in three different marine species that play a key role in marine/estuarine food webs and emphasize the relevance that metal speciation, specifically free metal ion concentration and activity, plays on this field of aquatic ecotoxicology.

As mentioned in the Introduction (**Chapter 1**), the current research aimed to respond to several questions initially raised, successfully address the set of objectives defined and present the most relevant results recorded in **Chapters 2** to **5**. In this last chapter, the main findings and most relevant conclusions reported on this PhD thesis are summarized and an approach to assess possible health implications with the use of Targent Hazard Quotients (THQ) and Estimated Weekly Intakes (EWI) is also presented. Furthermore, future perspectives will be addressed as well.

The effects of cadmium, based on free metal ions concentration, on life cycle parameters of the three species employed in this study, the copepod *Acartia tonsa*, the shrimp *Palaemon varians* and the fish *Solea senegalensis*, are presented and discussed in **Chapter 2**. The results showed that the highest sensitivity to cadmium is observed in *A. tonsa*, with the most sensitive endpoint being the Larval Development Ratio (LDR) of nauplii to copepodites. Sole eggs showed the highest tolerance to cadmium when compared to the other endpoints evaluated for all tested species. The cadmium toxicity

recorded was, by increasing order: *S*. *senegalensis* eggs < *P*. *varians* post-larvae < *P*. *varians* zoea I < *S*. *senegalensis* larvae < *A*. *tonsa* eggs < *A*. *tonsa* LDR. The higher tolerance of sole eggs to cadmium, when compared to larval stages, is potentially owed to the existence of the external membrane surrounding the embryo and likely acting as a protective barrier. For the larval stages, sensitivity is potentially due to undeveloped detoxifying organs. DNA damage increased in all species exposed to increasing cadmium concentrations, showing that cadmium is genotoxic to all three species tested and may impact not only individuals but also populations of marine taxa. Comparing all the above endpoints, sensitivity between species showed that *A. tonsa* again appears to be more sensitive to cadmium when compared to *P. varians* and *S. senegalensis*, thus suggesting a potential use as sentinel species for environmental contamination monitoring. To date, the use of metal speciation for the assessment of metal toxicity in marine organisms is not a common approach, thus the present work can be considered as a novel approach and set the base for future studies. In conclusion, in order to accurately evaluate the effects of cadmium in marine ecosystems, it is of great importance to understand its speciation.

In **Chapter 3**, the bioconcentration potential of cadmium under different environmental conditions was assessed by using toxicokinetic studies. The cadmium concentration selected was obtained based on tests previously performed in **Chapter 2**. Varying levels of pH, salinity and temperature showed to have a significant influence on metal accumulation by the copepod *A. tonsa*, mainly due to metabolic processes; cadmium speciation was only affected by salinity, as cadmium ion activity increased with decreasing salinity.

Cadmium concentration in the copepod increased with increasing pH, registering a peak at the intermediate pH of 7.5; it increased with increasing temperature and decreased with increasing salinity. The Biotic Ligand Model (BLM) theory provided an explanation for the pH pattern, as with decreasing pH, the $H^+$ activity increases and the negatively charged groups decrease by protonation, thus resulting in a competition between $H^+$ and $Cd^{2+}$ at the membrane binding sites. At the same time, temperature increased the metabolic rate, which can increase respiration rates, thus enhancing cadmium uptake. The copepod's physiology played a significant role for the explanation of the pattern observed with decreasing salinity, as in order to maintain osmotic balance at lower salinities through a possible increase of $Ca^{2+}$ uptake, resulted to the opportunistic transfer of $Cd^{2+}$ through the same channels. The high capacity of accumulating cadmium by the marine copepod *A. tonsa* under each metal-environmental condition may eventually pose a threat to

marine/estuarine trophic webs and therefore trophic transfer. In this way, possible biomagnification should be considered.

In **Chapter 4**, postlarval shrimp were exposed to cadmium concentrations through contaminated water, contaminated diet and a worst-case scenario where both contaminated water + diet were used; the kinetic parameters driving cadmium accumulation in the *P. varians* were estimated using two different versions of first-order toxicokinetic models. Results from this study suggested that at the end of the uptake phase, cadmium concentration in postlarval shrimp was the highest under the worst-case scenario. Cadmium uptake rate through water was faster when compared to the uptake rate from diet. In addition, shrimp were unable to eliminate cadmium from their body, showing no depuration rates in all three different exposure routes when modeled with the classic first-order one-compartment model. This result suggests that cadmium is possibly being stored in the shrimp. Therefore by including a model which considers the existence of an inert fraction in the organism confirmed the possible existence of a stored fraction, where cadmium is being accumulated and possibly detoxified, but not eliminated by the shrimp. In crustaceans, such storage is considered to mainly take place in the midgut gland, as trace metals tend to accumulate in this organ. Through the enhanced capacity of *P. varians* to uptake and store metal, it may be foreseen that in time their internal concentration may reach above the organisms' threshold and eventually promote the occurrence of mortality.
Biomagnification factors estimated by **Model 2** in the exposures through contaminated diet and both contaminated water + diet, were lower than unity signifying that cadmium concentration did not increase from prey to the predator. However, **Model 1** estimated that no steady state is reached, as internal concentration will keep increasing. Therefore it is uncertain whether biomagnification may occur, as it will depend on the critical body residue (CBR) of the organism that will lead to mortality. If the critical body residue in the organism is lower than the concentration in the prey then no biomagnification may occur.

Lastly, in **Chapter 5**, the accumulation of cadmium was assessed with the use of the benthic fish *Solea senegalensis* as a model species representing a higher trophic level and employing both contaminated water and diet (the ragworm *Hediste diversicolor*). No steady state was achieved after 14 days of exposure indicating that longer exposure periods are necessary to achieve an equilibrium between uptake and depuration. Muscle tissue showed a considerably low cadmium accumulation over 14 days of uptake, while

the highest accumulation value was recorded in the intestine. The highest metal uptake flux was observed in the intestine, resulting from the direct contact and assimilation of the metal from the contaminated diet (*H. diversicolor*) and the high drinking rate exhibited by marine fish (due to their hypertonic environment). The liver showed a decreased capacity in eliminating cadmium in the course of time, thus suggesting it can play the role of a storage compartment for cadmium during uptake and depuration. Low cadmium depuration rate and constant cadmium concentration in the gills, indicates that the metal is possibly stored in chloride cells due to its high affinity and similarity to $Ca^{+2}$, the most common entrance pathway for cadmium to cellular membranes.
As soles are consumed as food by humans, Target Hazard Quotients (THQ) were calculated for all age groups. Calculated values (<1) indicated that no risk arises for humans consuming fish with cadmium concentrations within the levels recorded in **Chapter 5** for muscle tissues. Nonetheless, it should be highlighted that time is a key issue and as mentioned above, increasing exposure time may lead to higher metal concentrations in the fish body and eventually pose a threat to human health.

Overall, it has been shown that cadmium is toxic to all marine/estuarine species evaluated in the present study. The copepod *A. tonsa* was the most sensitive among all species tested and, under the same exposure conditions as the shrimp *P. varians* and the fish *S. senegalensis*, it tends to accumulate higher levels of cadmium in its body. Deleterious effects promoted by cadmium contamination may therefore trigger bottom-up cascading effects, as *A. tonsa* occupies a low trophic level in marine food webs.

## 6.2. Implications to human health

Knowing the levels of bioaccumulation of a certain substance in an organism destined for human consumption is a useful and important step when assessing potential target hazard quotient and human health (Li et al., 2013; Sarkar et al., 2016). The human population is permanently exposed to an indirect contamination by cadmium through the consumption of fish and crustaceans, which may be higher in populations where these food items are more readily available and/or more consumed. Seafood is acknowledged as an important component of our diet due to their high content of essential elements (e.g. zinc) and vitamins (e.g. D, $B_{12}$, $B_6$) (Jakimska et al., 2011; Sarkar et al., 2016). Therefore, the consumption of marine organisms, such as fish and shrimp, which contain increased concentrations of a toxicant, can raise the levels of those metals in human tissues. Given

the fact that data acquired in this thesis can represent a source of information in human health risk, an attempt was made to estimate the THQ of cadmium and Estimated Daily Intake by consumption of shrimp using the metal load measured in **Chapter 4** and based on the same formulas used in **Chapter 5** for estimating EDI and THQ in fish.

The Estimated Weekly Intake (EWI) of cadmium was calculated using the following equation:

$EWI = \frac{Cm*WIR}{BW}$ (**Equation 6.1**)

where, $C_m$ is the metal concentration in shrimp after 4 days in mg $kg^{-1}$,

WIR is the weekly food ingestion rate in kg $week^{-1}$ $person^{-1}$ (0.150 for the Mediterranean population having 7 servings per week and 0.021 for having 1 serving per week (Losasso et al., 2015)), and

BW is the body weight in kg.

For the estimation of THQ for potential non-carcinogenic effects of cadmium in human health (United States Environmental Protection Agency (US EPA), 2010):

$THC = \frac{EF*ED*FIR*C_m}{RfD*BW*AT}$ (**Equation 6.2**)

where, EF is the exposure frequency in days $year^{-1}$ (365 days $year^{-1}$ for 7 servings per week and 52 days $year^{-1}$ for 1 serving per week (Copat et al., 2013)),

ED is the exposure duration in years,

FIR is the food ingestion rate in kg $day^{-1}$ $person^{-1}$ (0.021 for the Mediterranean population (Losasso et al., 2015)),

$C_m$ is the metal concentration in fish at steady state in mg $kg^{-1}$,

RfD is the reference dose in mg $kg^{-1}$ $day^{-1}$ (0.001 for cadmium (United States Environmental Protection Agency (US EPA), 2010)),

BW is the body weight in kg, and

AT is the average exposure time for non-carcinogens (365 days $year^{-1}$ x ED).

In the current study we used the same exposure duration values and body weight as Vieira et al (2011) according to US EPA (2008) for nine different age groups: 1 year and 14 kg (ages 1 to 3 years), 4 years and 21 kg (ages 4 to 6), 7 years and 32 kg (children 7 to 10 years), 11 years and 51 kg (adolescents 11 to 14 years), 15 years and 67 kg (adolescents 15 to 19 years), 20 years and 72 kg (adults 20 to 24 years), 25 years and 77 kg (adults 25 to 54 years), 55 years and 77 kg (adults 55 to 64 years) and 66 years and 72 kg (seniors >65 years).

**Table 6.1** Risk assessment for non-carcinogenic effects in humans by cadmium measured in the muscle of juvenile *Solea senegalensis* and the whole body of *Palaemon varians* using Estimated Weekly Intakes (EWI) (μg $kg^{-1}$ body weight) and Target Hazard Quotients (THQ) for different age groups. Provisional Tolerable Weekly Intake (PTWI) of cadmium is set at 2.5 μg $kg^{-1}$ body weight by EFSA (2012).

| | ***Solea senegalensis*** | | | | | | | | ***Palaemon varians*** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Age Group** | **EWI (1)** | **THQ (1)** | **EWI (2)** | **THQ (2)** | **EWI (3)** | **THQ (3)** | **EWI (4)** | **THQ (4)** | **EWI (5)** | **THQ (5)** | **EWI (6)** | **THQ (6)** |
| Children 1-3 yrs | 0.050 | 0.007 | 0.234 | 0.033 | 0.334 | 0.048 | 1.559 | 0.223 | **46.075** | **6.852** | **6.582** | 0.938 |
| Children 4-6 yrs | 0.033 | 0.005 | 0.156 | 0.022 | 0.223 | 0.032 | 1.039 | 0.148 | **30.716** | **4.388** | **4.388** | 0.625 |
| Children 7-10 yrs | 0.022 | 0.003 | 0.102 | 0.015 | 0.146 | 0.021 | 0.682 | 0.097 | **20.158** | **2.880** | **2.880** | 0.410 |
| Adolescents 11-14 yrs | 0.014 | 0.002 | 0.064 | 0.009 | 0.092 | 0.013 | 0.428 | 0.061 | **12.648** | **1.807** | 1.807 | 0.257 |
| Adolescents 15-19 yrs | 0.010 | 0.001 | 0.049 | 0.007 | 0.070 | 0.010 | 0.326 | 0.047 | **9.628** | **1.375** | 1.375 | 0.196 |
| Adults 20-24 yrs | 0.010 | 0.001 | 0.045 | 0.006 | 0.065 | 0.009 | 0.303 | 0.043 | **8.959** | **1.280** | 1.280 | 0.182 |
| Adults 25-54 yrs | 0.009 | 0.001 | 0.043 | 0.006 | 0.061 | 0.009 | 0.283 | 0.040 | **8.377** | **1.197** | 1.197 | 0.170 |
| Adults 54-64 yrs | 0.009 | 0.001 | 0.043 | 0.006 | 0.061 | 0.009 | 0.283 | 0.040 | **8.377** | **1.197** | 1.197 | 0.170 |
| Seniors > 65 yrs | 0.010 | 0.001 | 0.045 | 0.006 | 0.065 | 0.009 | 0.303 | 0.043 | **8.959** | **1.280** | 1.280 | 0.182 |

(1) THQ/EWI values according to World fish consumption using cadmium concentration in muscle after 14 days

(2) THQ/EWI values according to Portugal's fish consumption using cadmium concentration in muscle after 14 days

(3) THQ/EWI values according to World's fish consumption using cadmium concentration in muscle at steady state

(4) THQ/EWI values according to Portugal's fish consumption using cadmium concentration in muscle at steady state

(5) THQ/EWI values according to Mediterranean's shellfish consumption (7 days per week) using cadmium concentration in shrimp whole body

(6) THQ/EWI values according to Mediterranean's shellfish consumption (1 day per week) using cadmium concentration in shrimp whole body

As mentioned in **Chapter 5**, THQ values <1 to all age groups exposed in the cadmium concentration measured in sole's muscle indicated that the exposed population can be considered safe. However, THQ values ranging between 1 and 5 from consumption of

contaminated shellfish with the measured concentration in the shrimp body indicate that the population is in a level of concern when consuming this food item on a daily basis. Nevertheless, when consumption is limited to once a week the THQ values are <1, although the EWI is not within the provisional tolerable weekly intake for children under 10 yrs old, which would therefore increase the potential risk of cadmium in those specific age groups.

## 6.3. Final conclusion and future perspectives

The present PhD study validates important aspects on cadmium toxicity for marine species by adapting guidelines and methodologies of model species to the ones employed here and the evaluation of the potential transfer of cadmium across different marine trophic levels. Briefly, findings from the current thesis point out the relevance of assessing cadmium eco- and genotoxicity using cadmium speciation, an approach that is not often adopted when evaluating the effect of metals in marine ecotoxicology. As already highlighted by several authors (Mariani et al., 2006; Pérez et al., 2016; Raisuddin et al., 2007), this thesis stresses the need to establish clear guidelines for assessing toxic substances and reporting data in the marine/estuarine environment, as well as to introduce new protocols employing different taxa (e.g. marine fish) as model species in the marine environment. The use of different taxonomic groups spanning across multiple trophic levels is paramount to obtain a broader and more in depth knowledge of the true impact that contaminants have in marine/brackish ecosystems, as different organisms often display contrasting bioaccumulation mechanisms (Ardestani et al., 2014). Furthermore, this thesis demonstrated the relevance of toxicokinetic studies for evaluating the bioaccumulation potential and subsequently, the potential toxicity of cadmium in three distinct marine organisms under different environmental conditions and exposure routes. Ashauer and Escher (2010) have demonstrated that the mechanisms behind induced toxicity over time at an organismal level can be easily reproduced with the use of toxicokinetic-toxicodynamic (TK-TD) models; this approach provides the advantage of using a conceptual framework in order to better perceive the reason for high variability in the toxicity of different species exposed to the same contaminant.

In the future, it is important that researchers testing the toxicity of cadmium in the marine/estuarine environment employ a similar approach as the one presented in this study, but also include cadmium contaminated sediment when evaluating eco-, genotoxicity and bioaccumulation of this metal, cadmium. Sediments are the final sinks of

metals once they have entered the marine environment, thus making their survey in this type of studies environmentally relevant. Moreover, the use of biomarkers of exposure and effects would provide substantial information on the level of contamination and how organisms react at lower biological organizational levels. Biomarkers usually assessed for metal contamination provide information mainly related to oxidative stress, detoxification processes and genotoxicity and are i) metallothionein (MT) gene expression (binds to metals detoxifying them), ii) cytochrome P450 and EROD activity and iii) glutathione (GSH) (prevents damage to cellular components due to ROS induction), among several others. Therefore, the biomarker concept is considered an important approach in ecotoxicological studies and they are used as an early warning for assessing an ecosystem's health under environmental stress. Furthermore it is vital for the marine environment that whenever relevant toxicity and bioaccumulation of chemical mixtures e.g. estimate the bioaccumulation potential of cadmium under a simultaneous zinc exposure, should be considered as cadmium is a by-product from zinc mining and they are usually found together in the marine environment. In addition to this join stressors approach, cadmium combinations under a full factorial design of fluctuating abiotic factors, e.g. salinity, should also be considered.

In conclusion, the present study has provided a baseline that highlights the importance of establishing standardized protocols and guidelines for future experiments, in order to: i) assist on the planning and performance of more complex experiments (e.g. bioaccumulation studies using two exposure pathways, mixtures) and ii) assess metal toxicity in the marine environment in a uniform and systematic way, standardize data reporting and allow the use of meta-analysis to enhance our ability to compare multiple studies on a reliable way. In addition, and specifically for cadmium, a priority chemical in worldwide legislation/regulation, this study provided more scientific knowledge on cadmium hazard assessment which can be used within risk assessment procedures for marine environments. Finally, bridging the gap between effects to the environment and human health was also attained in the present work.

## 6.4. References

Ardestani, M.M., van Straalen, N.M., van Gestel, C.A.M., 2014. Uptake and elimination kinetics of metals in soil invertebrates: a review. Environ. Pollut. 193, 277–295. doi:10.1016/j.envpol.2014.06.026

Ashauer, R., Escher, B.I., 2010. Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. J Environ Monit 12, 2056–2061. doi:10.1039/c0em00234h

Copat, C., Arena, G., Fiore, M., Ledda, C., Fallico, R., Sciacca, S., Ferrante, M., 2013. Heavy metals concentrations in fish and shellfish from eastern Mediterranean Sea: Consumption advisories. Food Chem. Toxicol. 53, 33–37. doi:10.1016/j.fct.2012.11.038

European Food Safety Authority (EFSA), 2012. Cadmium dietary exposure in the European population, EFSA Journal. Eur Food Saf Authority (doi:10.2903/j.efsa.2012.2551

Jakimska, A., Konieczka, P., Skóra, K., Namiesnik, J., 2011. Bioaccumulation of metals in tissues of marine animals. Part I: The role and impact of heavy metals on organisms. Pol J Environ Stud 20, 1117–1125.

Li, J., Huang, Z., Huang, Z.Y., Hu, Y., Yang, H., 2013. Potential risk assessment of heavy metals by consuming shellfish collected from Xiamen, China. Environ Sci Pollut Res Int 20, 2937–2947. doi:10.1007/s11356-012-1207-3

Losasso, C., Bille, L., Patuzzi, I., Lorenzetto, M., Binato, G., Dalla Pozza, M., Ferrè, N., Ricci, A., 2015. Possible influence of natural events on heavy metals exposure from shellfish consumption: a case study in the north-East of Italy. Front Public Health 3, 21. doi:10.3389/fpubh.2015.00021

Mariani, L., De Pascale, D., Faraponova, O., Tornambè, A., Sarni, A., Giuliani, S., Ruggiero, G., Onorati, F., Magaletti, E., 2006. The use of a test battery in marine ecotoxicology: The acute toxicity of sodium dodecyl sulfate. Environ. Toxicol. 21, 373–379. doi:10.1002/tox.20204

Mhadhbi, L., Boumaiza, M., Beiras, R., 2010. A standard ecotoxicological bioassay using early life stages of the marine fish *Psetta maxima*. Aquatic Living Resources 23, 209–216. doi:10.1051/alr/2010014

Pérez, S., Fernández, N., Ribeiro, P.A., 2016. Standardization of a *Patella spp*. (Mollusca, Gastropoda) embryo–larval bioassay and advantages of its use in marine ecotoxicology. Ecotoxicol Environ Safety 127, 175–186.

doi:10.1016/j.ecoenv.2016.01.025

Raisuddin, S., Kwok, K.W.H., Leung, K.M.Y., Schlenk, D., Lee, J.-S., 2007. The copepod *Tigriopus*: a promising marine model organism for ecotoxicology and environmental genomics. Aquat Toxicol 83, 161–173. doi:10.1016/j.aquatox.2007.04.005

Sarkar, T., Alam, M.M., Parvin, N., Fardous, Z., Chowdhury, A.Z., Hossain, S., Haque, M.E., Biswas, N., 2016. Assessment of heavy metals contamination and human health risk in shrimp collected from different farms and rivers at Khulna-Satkhira region, Bangladesh. Toxicology Reports 3, 346–350. doi:10.1016/j.toxrep.2016.03.003

United States Environmental Protection Agency (US EPA), 2010. Risk-Based Concentration Table [WWW Document]. http://www.epa.gov/reghwmd/risk/human/index.htm).

United States Environmental Protection Agency (US EPA), 2008. Child-Specific Exposure Factors Handbook (Final Report) (No. EPA/600/R-06/096F). Washington, DC.

Vieira, C., Morais, S., Ramos, S., Delerue-Matos, C., Oliveira, M.B.P.P., 2011. Mercury, cadmium, lead and arsenic levels in three pelagic fish species from the Atlantic Ocean: Intra- and inter-specific variability and human health risks for consumption. Food Chem. Toxicol. 49, 923–932. doi:10.1016/j.fct.2010.12.016

Wood, C.M., Farrell, A.P., Brauner, C.J., 2012. Homeostasis and Toxicology of Essential Metals. Academic Press.